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BIOCHEMISTRY AND PHARMACOLOGY OF COMPOUNDS DERIVED FROM MARINE ORGANISMS

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CONTENTS

Perspectives in Marine Biochemistry. *By* Denis L. Fox 617
An Investigation of the Distribution and Function of Homarine (*N*-Methyl Picolinic Acid). *By* E. L. Gasteiger, P. C. Haake, and J. A. Gergen 622
Pharmacologically Active Amines and Their Biogenesis in the Octopus. *By* William J. Hartman, William Gilbert Clark, Shirley D. Cyr, Albert L. Jordon, and Ruth A. Leibhold 637
Some Chemical Properties of Nereistoxin. *By* Yoshiro Hashimoto and Tomotoshi Okaichi 667
Salivary Poison of a Marine Gastropod, *Neptunea arthritica* Bernardi, and the Seasonal Variation of Its Toxicity. *By* Motokazu Asano and Masao Itoh 674
The Salivary Gland of *Neptunea antiqua*. *By* Ragnar Fänge 689
Pharmacologically Active Choline Esters in Marine Gastropods. *By* V. P. Whittaker. 695
Preliminary Studies on the Venom of the Marine Snail *Conus* *By* A. J. Kohn, P. R. Saunders, and S. Wiener 706
Toxicity of Octopus Saliva Against Crustacea. *By* F. Ghiretti 726
The Toxin of *Physalia* Nematocysts. *By* Charles E. Lane 742
Mechanism of Action of a Histamine-Liberating Principle in Jellyfish (*Cyanea capillata*). *By* Börje Uvnäs 751
Antibodylike Materials of Marine Invertebrates. *By* John H. Phillips 760
Observations on Ciguatera-Type Toxin in Fish. *By* Albert H. Banner, Paul J. Scheuer, Satoshi Sasaki, Philip Helfrich, and Charles B. Alender 770
Marine Biotoxins. I. Ciguatera Poison: Some Biological and Chemical Aspects. *By* Donald W. Hessel, Bruce W. Halstead, and Norman H. Peckham 788

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Pharmacological and Chemical Studies of the Venom of the Stonefish (Genus *Synanceja*) and Other Scorpionfishes. *By* Paul R. Saunders 798
Venom of the Weevers *Trachinus draco* and *Trachinus vipera*. *By* Findlay E. Russell and Jerry A. Emery 805
Pharmacological Study of Poisons from Shellfish and Puffer Fish. *By* Edmund F. Murtha 820
Analysis of the Toxin Produced by *Gonyaulax Catenella* in Axenic Culture. *By* John M. Burke, John Marchisotto, John J. A. McLaughlin, and Luigi Provasoli 837
Biochemical Studies on Paralytic Shellfish Poisons. *By* Edward J. Schantz 843
Mass Cultivation of Some Phytoplanktons. *By* John J. A. McLaughlin, Paul A. Zahl, Andrew Nowak, John Marchisotto, and Jan Prager 856
Studies on the Toxic Principles Formed by the Chrysomonad *Prymnesium parvum* Carter. *By* Moshe Shilo and Robert F. Rosenberger 866
Steroids in Marine Organisms. *By* G. A. D. Haslewood 877
Effects of Holothurin, a Steroid Saponin from the Bahamian Sea Cucumber (*Actinopyga agassizi*), on Various Biological Systems. *By* Ross F. Nigrelli and Sophie Jakowska 884
Some Pharmacologic Properties of Holothurin A, a Glycosidic Mixture from the Sea Cucumber. *By* S. L. Friess, F. G. Standaert, E. R. Whitcomb, R. F. Nigrelli, J. D. Chanley, and Harry Sobotka 893
Further Studies on the Structure of Holothurin. *By* J. D. Chanley, J. Perlstein, Ross F. Nigrelli, and Harry Sobotka 902
Sterols of Some Invertebrates. *By* Werner Bergmann and Irving I. Domsky 906
The Nucleic Acids of Sponges. *By* Martin F. Stempien, Jr. 910
Antimicrobial Substances from Sponges. *By* Sophie Jakowska and Ross F. Nigrelli. 913
Chemistry of Coelenterates. I. Occurrence of Terpenoid Compounds in Gorgonians. *By* Leon S. Ciereszko, Dewey H. Sifford, and Alfred J. Weinheimer 917
Chemistry of Coelenterates. II. Occurrence of Taurobetaine and Creatine in Gorgonians. *By* Leon S. Ciereszko, Paul H. Odense, and Robert W. Schmidt 920
Phosphagens of Marine Animals. *By* Nguyen van Thoai and Jean Roche 923
Histoenzymological Data on the Digestive Tract of *Octopus vulgaris* Lamarck (Cephalopoda). *By* Lucie Arvy 929

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PERSPECTIVES IN MARINE BIOCHEMISTRY

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How far back into time should one try to retreat, and then where should he endeavor to focus his sights on the vast marine world in order to present a few guiding perspectives in marine biochemistry? Different biologists or biochemists would react to these queries with their own various individual responses. I shall try to present a few basic panoramic views for your thinking and criticism.

The marine environment has, since primordial times, provided a moist, chemically polar reaction-medium containing dissolved electrolytes and characterized by conditions of temperature, movement, and innumerable diversified interfaces, all favorable to many kinds of prebiochemical events and syntheses. Moreover, there were the factors of time and the mass law, favoring reactions between molecules adsorbed to and thus concentrated at various kinds of inorganic colloidal and other surfaces; also there were general screening or buffering effects against sudden photic, thermal, chemical, or mechanical changes bearing upon labile compounds. Such features doubtless would have promoted marine waters as the medium of the earliest organisms and, as time passed, an environment wherein the widest permutations could occur, leading toward the greatest variations in size, shape, constitution, and functions of evolved living things.

The marine milieu and its enormous diversity of inhabitants must be regarded as having evolved together. Today the total surface area of the world's oceans exceeds that of the exposed land by about 2.4-fold, while the estimated *volume* of the marine biocycle bears to the combined volumes of terrestrial and fresh water biocycles a ratio of about 300:1.[1] And the greatest diversity and numbers of animal species are still to be found in the oceans. Indeed, several whole groups are exclusively marine, for example, echinoderms and tunicates, amphineuran, scaphopod, and cephalopod mollusks, brachiopods and gephyrean worms, while few save the insects and amphibian vertebrates are to be regarded as strictly nonmarine classes.

The oceans themselves gradually increased not only in volume but in concentrations of dissolved gases and salts; the concentrations and ratios of ions in today's marine, fresh-water, and terrestrial vertebrates are regarded as reflecting closely the electrolytic conditions prevailing in the seas at the time of the ancestral vertebrates' emergence in the evolutionary scale.[2]

The total mass of loose parts lying about has always exceeded that of the finished products entering the evolutionary procession; and while there is no estimating either the diversity of kinds or the preponderance in weight or numbers of inanimate organic molecules over reproducing entities at any past era in time, it should be useful to review a few conservative assumptions about qualitative diversity and relative concentrations in today's seas.

It is logical to regard abiogenesis as having had its day long ago, since the current world harbors organisms of incalculably diversified biochemical habits and capacities, ready to assimilate any simple organic molecules that conceivably might emerge to build their own way toward systems of greater complexity. Hence all organic detritus of today's world (discounting a few metallic carbides, cyanide minerals, and the like) is of direct biological origin, whether from dead bodies of plants, animals, and microbes, or from their excreted or otherwise discarded materials. While the chemical variety of such organic molecules obviously must be very large, the present-day assortment can offer no challenge to compare with the vast assemblage and diversification that must have prevailed in prebiological times. Nevertheless, the ever-present unorganized, nonliving material, for example, in the oceans of today, constitutes the basic source of food supply for countless billions of omnivorous marine animals, not to mention, for the moment, the biomass of bacterial and fungal flora. This is true whether the detritophagous feeders obtain their food by ciliary, mucous, or setous filtration, or through combinations of these; by scraping adsorbed films of slime from immersed surfaces, or by ingesting masses of bottom sand, mud, or other relatively fine material to obtain therefrom associated organic matter, as does the earthworm in land soils. All of this applies also whether the creatures elaborate and secrete all of their own necessary digestive enzymes or, alternatively, like some land forms, harbor in their alimentary tracts specific microflora capable of hydrolyzing certain of the more refractory components. Indeed, this field is a marine nutritional area concerning which little indeed is yet known and therefore calls urgently for serious biochemical and microbiological attention.

Let us for the present exclude consideration of the strict carnivores, for example, the coelenterates, asteroid echinoderms, cephalopod mollusks, and many of the fishes, as well as the exclusively herbivorous species that devour fresh seaweed material, as do numerous gastropods and certain fishes. Let us contemplate instead some true scavengers, or feeders largely upon inanimate organic detritus.[3]

Filter feeders, removing from the water and swallowing marine leptopel, that is, colloidal and other finely particulate organic matter, not excluding adsorbed bacteria and other microscopic organisms, are represented among the setous screeners, such as barnacles, copepods, and some fishes supplied with gill-rakers. The mucociliary operators, elaborating sheets of mucus over extensive surfaces supplied with countless fine, hairlike cilia sweeping the mucus and its adsorbed complement of detritus toward the mouth, are found among the bivalve mollusks, such as oysters, clams, and mussels, as well as tunicates, crinoid echinoderms, and many microcrustaceans; the latter group may combine the setous and mucous functions.

So-called scrapers are represented chiefly by marine snails bearing a tough, horny tongue or radula, operated as a kind of rasp by strong buccal muscles that often exhibit the red colors of hemoglobin. True, the scrapers inevitably consume both plant and small-animal organisms along with the slimy inanimate component, just as filter-feeders swallow many such microorganisms with the leptopel. A few primitive animals are provided with sucking mechanisms for removing the so-called sapropel, pelogloea, or muddy slime.

Finally, the shovelers propel whole mud or sand into the mouth by pharyngeal muscular action (such as in many worms), by circumoral tentacles supplied with adhesive, distal pads (in some holothurians), by adhesive walking feet and seizing mouth-parts (of ophiuroids), or by tubular, sucking mouths, as in some bottom-feeding fish that consume sapropel, the viscous, slimy carpet of organic detrital material covering the bottom surface of some regions.

To achieve some general idea of the vast amounts of organic leptopel suspended in ocean waters, we may take a conservative figure of 1 ppm* as its average, over-all concentration, omitting the substantial fractions adsorbed to solid surfaces as pelogloea or resting on the floors as sapropel, and notably the large concentrations of organic matter that characterize foreshores the world over. Assigning the organic leptopel a density value approximating that of water (62.4 lb./cu. ft.) and, using the accepted value of 330×10^6 mi.^3 as the total additive volume of the world's oceans, we obtain a product of $(5280)^3 \times 330 \times 62.4 = 31 \times 10^{14}$ lb., or 1.5×10^{12} tons of suspended organic matter, serving directly or potentially, via bacteria, as a raw food source in the perpetual metabolic cycles in the marine world. The figure exceeds by a whole order of magnitude the estimated value of 1.5×10^{11} tons of organic matter synthesized annually by marine phytoplankton, as derived from surveys by Riley and others.[9,10] And this latter value, in turn, exceeds by nearly tenfold the estimated total annual productivity of land plants, and surely by a greater ratio the total mass of animal life. Since plants are the primary producers in any community, a comparison between the foregoing estimates emphasizes the conclusion that the standing crop of organic leptopel must exceed by at least tenfold (perhaps 100-fold) the annual synthesis or standing crop of total living marine organisms.

Let us now look for a moment at the nutritional activities of three representative marine detritus feeders: (1) the California mussel, *Mytilus californianus*, representing the filterers; (2) an intertidal polychaete worm, *Thoracophelia mucronata*, characterizing the shovelers or wholesale swallowers of finely divided bottom material; and (3) a common, intertidal periwinkle, *Littorina planaxis*, as an illustration of the scrapers.

Earlier researches at La Jolla[3] indicate that a moderate colony of 10^6 mussels will, during their second year, filter the leptopel from some 22×10^6 tons of environmental water, or from a volume equivalent to a stratum 1 mile square by 25 feet deep. If the organic matter suspended in this near-shore water amounts to a year-round, average value of 1.5 ppm, the mollusks must remove about 33 tons of such material. The animals gain collectively about 4 tons of tissue (on a dry, ash-free basis), thus indicating an "efficiency" or ratio of assimilated to ingested material of about 12 per cent. Moreover, since considerable proportions of the available organic material are relatively inert and indigestible, such as chitin, cellulose, humus, and similar refractory products, and since a part of the total may be adsorbed to inorganic bodies rejected by the animal due to large size or rough shape, the above figure, in all probability, is low. Indeed, the efficiency of these mussels, based upon dry, ash-free organic weight-increment of new tissue and dry, ash-free content of intaken material as measured in the feces, gives the high but still probably conservative quotient of 17.6 per cent.

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* Concentrations vary widely with latitude,[3] depth, season, distance from shore, and biochemical activity; deep waters (600 m.) may yield as little as 0.25 ppm, while surface waters, notably in the area of marine slicks, may contain 140-fold as much.

A beach sand bed occupying 1 square mile, aggregating 500 miles in length, 10 feet wide and 1 foot deep, harboring the burrowing annelid *Thoracophelia* in populations of about 3000 per square foot, will therefore contain approximately 8×10^{10} individuals weighing an aggregate of 3500 tons, and will amount to 1.4×10^{6} tons of sand (dry weight), involving hardly more than 1 per cent organic matter. The worms, swallowing 2100-fold their own weight of sand annually, will therefore cycle about 7.3×10^{6} tons of their substrate each year, or the entire quantity of sand in the total worm-bed once every 10 weeks. Of the estimated 7×10^{4} tons of organic matter available in the sand, the worms will convert 700 tons into new tissue, assuming an annual life cycle. A two-year life cycle, believed to be more likely in this species, would therefore indicate an efficiency of only about 0.5 per cent for these representative, relatively wasteful scavengers.

Turning to the third example, the intertidal gastropod *Littorina*, one finds that this little periwinkle achieves a much higher efficiency than that calculated for the annelid worm, and rather close to that found in the mussel. It scrapes and swallows impacted Eocene and Cretaceous siltstone bearing attached algae and pelogloeal materials in quantities amounting to about 2.6 per cent of organic matter by weight. An estimated population of 8.6×10^{8} snails on a square mile of wet siltstone will remove approximately 2200 tons annually, fixing as new tissue some 6.82 of the total 57.2 tons of organic matter available, thus demonstrating an efficiency of nearly 12 per cent.

So much for the numerically great but proportionately infinitesimal fractions of marine organic detritus cycled by these scavengers. It may be of additional interest to review the rather contrasting metabolic fractionation of certain known molecular constituents (that is, the carotenoids) of the omnivorous and, for practical purposes, almost identical basic diet of these and some other detritophagous marine animals.

Marine detritus, whether suspended in the upper waters as leptopel or adsorbed to solid interfaces as pelogloea, contains both the hydrocarbon (prominently β- and secondarily α-carotenes) and alcoholic types (various algal xanthophylls) of polyene pigments characteristic of phytoplankton and other marine plants. In plant cells, in the detritus derived largely therefrom, and in the majority of marine animals, the xanthophylls preponderate greatly over the carotenes, for example, by ratios of 9:1 or greater in plants, while some animals store exclusively members of the xanthophyll class. But there are exceptions among the animals, of which the worm *Thoracophelia* happens to be an outstanding one, since it not only assimilates selectively the hydrocarbon type of carotenoid, but solely the β-carotene, rejecting the α-isomer; moreover, the xanthophylls not only are not assimilated as such, but are destroyed during the brief interval during which the sand traverses the length of the worm's alimentary tract. *Thoracophelia* stores several fluorescent chromolipoids and is believed to employ active lipoxidases in its metabolism of fat-soluble materials.[4]

The mussel manages a contrasting biochemical performance, completely rejecting the dietary carotenes, assimilating the xanthophylls and converting some fraction of the carotenoid intake, presumably by oxidation, into a new acidic pigment, named mytiloxanthin by Scheer,[5] who discovered and isolated the compound in my laboratory many years ago.

The periwinkle *Littorina* seems, by contrast with the foregoing species, to be relatively conventional and unoriginal, storing what appears to be a full complement of the carotenes and algal xanthophylls from its natural diet.[6] The sea-cucumber *Stichopus parvimensis*, feeding as does *Thoracophelia* upon sand grains thinly coated with pelogloeal matter, is rather unselective of the kinds of lipochromes assimilated, storing members of both chief classes, although in very small quantities, while other echinoderms consuming bottom detritus, such as the ophiuroids or serpent stars *Ophiopterus papillosa*, *Ophiothrix spiculata*, and *O. rudis*, store no carotenes whatever, but only free and esterified xanthophylls, including several unique, very labile members.[7] Pelagic crustaceans, including euphausiids and some copepods, feeding upon leptopel, convert part of the ingested carotenoid material into the red, acidogenic, ketoxanthophyll astaxanthin. In addition, numerous euphausiids contain vitamin A derived from carotene or, perhaps in some instances, from astaxanthin, concentrated largely in the eye stalks.[8]

These few vignettes will, it is hoped, serve to emphasize the vast wealth of marine biochemical problems and the great contrasts in the nutritional habits of marine animals and in their biochemical metabolism of labile, colored molecules of the carotenoid group, obtained originally from plants. One hardly may wonder at the differences in metabolic efficiency, enzyme action, and the rich assortment of endogenous nitrogen compounds, some of them toxic, still encountered in the long evolutionary chains represented among marine animals.

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AN INVESTIGATION OF THE DISTRIBUTION AND FUNCTION OF HOMARINE (*N*-METHYL PICOLINIC ACID)*

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Introduction

During an investigation of the action of polychromatic ultraviolet (UV) light on the giant axon of squid,[1] the identification of UV-absorbing constituents of the nerve became an engrossing problem. It was hoped that such components, if destroyed by irradiation, would account for the changes in electrical properties of nerve that are produced by UV.

A. M. Shanes and T. J. DeKornfeld[2] had published absorption spectra of invertebrate nerve extracts, which showed the presence of a surprisingly strong absorber of UV light at 271 mμ. Experiments of H. M. Kalckar *et al.*[3] led them to suggest that this absorption peak was caused by homarine (*N*-methyl picolinic acid), a substance extracted by F. A. Hoppe-Seyler[4] (1933) from lobster. Homarine is a quaternary ammonium animal base with many interesting characteristics that suggest several possible roles in nerve function. It is found in high concentrations in nerve and is destroyed by UV irradiation. This led us to an extensive investigation of the distribution of homarine in tissues and phyla and to perfusion studies of the lobster heart with homarine and its possible precursors or breakdown products. The lobster heart was chosen because its neurogenic beat can serve as a convenient indicator of the pharmacological action of materials being considered.

Unfortunately, our original hope has not been realized, and it now seems unlikely that homarine is involved in membrane activity. In the irradiation experiments it appears instead to reduce the effectiveness of UV on the membrane by serving as a protective absorber. However, the resulting investigations reported here reveal many characteristics of this compound and suggest a possible role for it in cellular osmoregulation.

Characteristics of Homarine

Our identification of extracted homarine was based on comparison of the synthetic and extracted material with respect to peak position, shape, and *p*H dependence of their absorption curves, and behavior of the materials in paper and column chromatography.

The synthetic material was prepared‡ according to the method of Hoppe-Seyler,[4] while the extracted material was prepared by the chemical and ion exchange methods used by Kalckar and his associates.[3] In the final step, pure crystals were obtained from methyl alcohol. The chromatographic col-

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‡ Homarine and related compounds were kindly supplied by L. M. Long and M. L. Black of the Parke-Davis Company, Detroit, Mich.

umn was a cylinder (2.1 cm. diameter and 59 cm. long) of Dowex 50 resin, cross linkage 8×. The resin was processed to the acid state by washing it with 550 cc. of 1 *N* NaOH and 500 cc. of 2 *N* HCl. About 135 cc. of deproteinized crude extract of whelk (*Busycon canaliculatum*) in 0.2 *N* HCl was added, after which the column was washed with 2 *N* HCl at an approximate rate of 40 cc./hour. Fractions were retained when the homarine absorption spectrum could be detected in samples of the eluent.

A second method of comparing synthetic and extracted homarine was by running samples on Whatman No. 3 chromatographic paper in a solvent phase consisting of 95 per cent ethyl alcohol (95 per cent concentration) and 5 per cent ammonium hydroxide (concentrated), after the method of H. N. Bregoff *et al.*[5] The solvents moved in a descending direction. By use of Folin micropipettes, the paper was charged with 0.1 cc. of crude aqueous extract, as well as control samples of synthetic homarine, to facilitate the identification of the homarine spot. After 16 hours of running time the papers were dried, the homarine spots outlined visually under a UV lamp, and the materials eluted free by cutting around the spots and slowly washing them with 2 cc. of distilled water flowing from No. 27 needles.

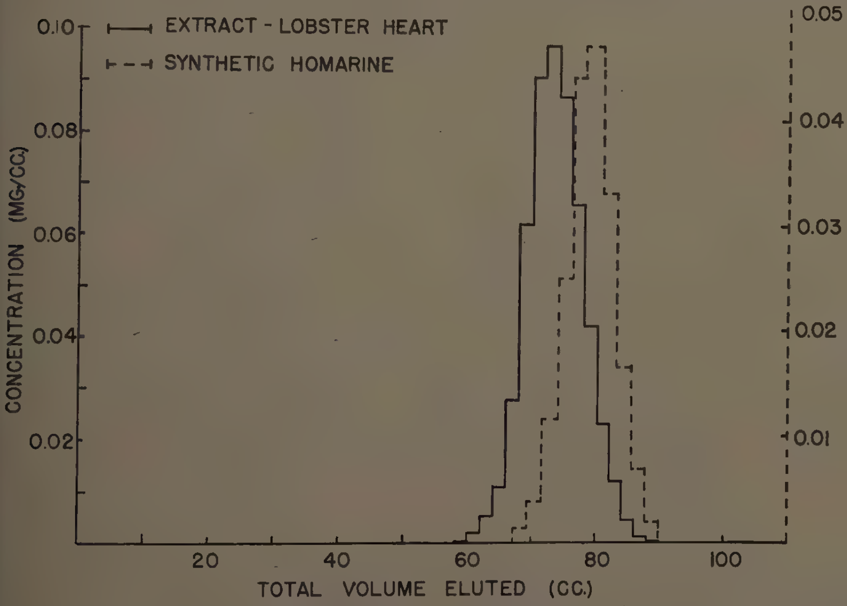

FIGURE 1. Comparison of synthetic and natural homarine eluted from a resin column. The fractions were assayed by UV absorption and plotted on the abscissa, with their corresponding concentrations plotted on the ordinate. The curves were normalized at their peaks.

In both column and paper chromatography there was good agreement between the handling of the synthetic and the extracted materials. FIGURE 1 illustrates the efflux of synthetic and natural material from the resin column; fractions plotted along the abscissa were assayed by means of UV absorption and are graphed here against the concentration in mg./cc. Since the column was not charged with identical concentrations in the two cases, the curves were normalized to one another at their peaks to permit a comparison of their shapes and time of appearance. The shapes of the graphs are nearly identical, while the synthetic appears to run slower than the extracted material. This difference can probably be explained as due to uncontrolled salts in the extract and variations in running rate and temperature that were not precisely controlled in the operation of the column.

Absorption spectra of synthetic and extracted homarine are illustrated in FIGURE 2. The absorption minimum is found at 240 mμ and the absorption maximum at 272 mμ. Changing the *p*H of the solution from acid to base shifts the peak for the synthetic and the extracted materials about 2.5 mμ toward the longer wave lengths. These curves and their *p*H dependence are in excellent agreement with those obtained by T. Hultin *et al.*[6] The methylation of α-picolinic acid to form homarine can be monitored by following the shift and increase in amplitude of the 265-mμ absorption peak of α-picolinic acid to the 272-mμ peak of homarine. UV irradiation of the synthetic and purified extracts destroyed the material, as seen from the reduction of the 272-mμ peak as the irradiation progressed (FIGURE 3). There was no obvious shift of the peak position for the synthetic homarine, although a small shift

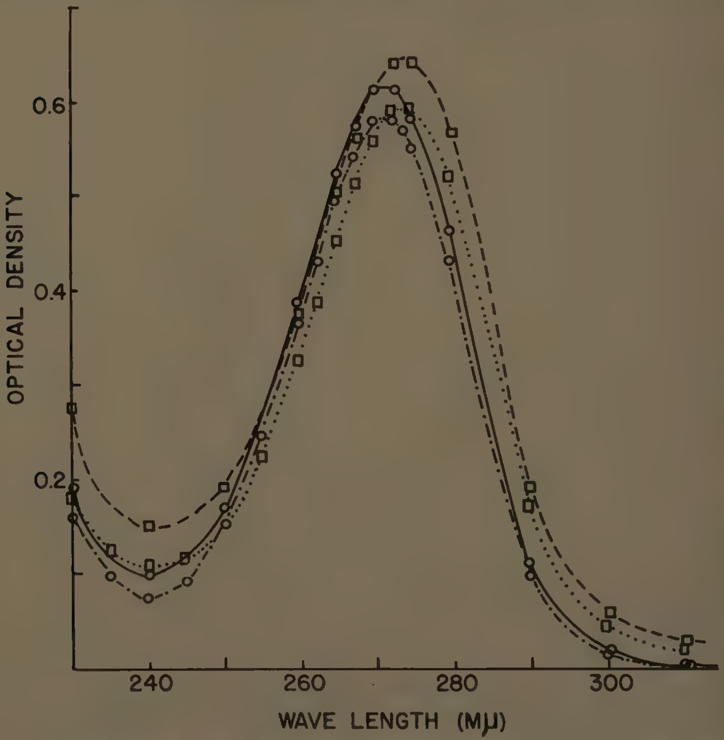

FIGURE 2. Comparison of the absorption spectra of synthetic and extracted homarine in acidic and basic solutions. Key: ○—○ extracted homarine in 0.5 *M* HCl; □- - -□ extracted homarine in 0.3 *M* NaOH; ○-·-○ synthetic homarine in 0.5 *M* HCl; and □···□ synthetic homarine in 0.3 *M* NaOH.

towards 265 mμ was occasionally seen for the purified extracts. This shift was probably due to an increase in the background absorption of the less pure extracts. Similar reduction of the 272-mμ absorption peak was seen after irradiation of functioning giant axons. No change in the absorption spectrum was found upon heating homarine in solution to 100° C. for 20 min.

Two determinations of the molar extinction coefficient were made, one giving a value of 6370 for the material synthesized in our laboratory and the second giving a value of 6280 for the material synthesized at Parke-Davis Company. These values agree within 2 per cent of one another, giving an average of 6325 l./mole/cm.

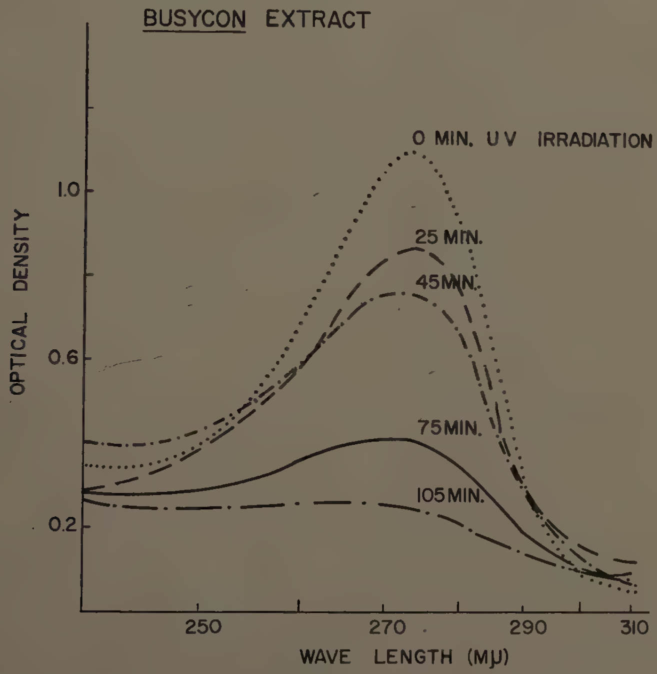

Figure 3. Destruction of homarine by UV irradiation. The sample was extracted from *Busycon* muscle by paper chromatography.

Biological Activity of Homarine

Because of our interest in nerve and the promise of a pharmacological role in excitable tissue, homarine and related compounds were perfused through the beating lobster heart. This preparation was made according to the method of J. H. Welsh.[7] The heart was cannulated through the dorsal abdominal artery and perfused backward, with the perfusion fluid[8] flowing out through the ostia. The ECG was recorded across the cardiac wall by inserting one Ag-AgCl electrode inside the perfusion cannula near its tip and another in the bath surrounding the heart. In order to record the mechanogram, the heart was attached to the end of an isometric lever that consisted of a 1⅜″ ×

¾″ tongue of 1/32″ Al, with four strain gauge elements affixed near the junction of the tongue and its larger supporting section. The mechanogram and ECG were amplified and recorded by means of a Sanborn two-channel system.

Solutions were prepared for perfusion by titrating with NaOH to *p*H 7.2 to 7.6 and correcting for the NaCl, which was formed upon the addition of the base. This variation in *p*H did not affect the amplitude and frequency of beat, an observation made earlier by W. H. Cole,[9] who reported no changes over a *p*H range from 7.0 to 8.0.

After appropriate control periods, 25 to 100 cc. of test solution were perfused through the heart over a period of 4 to 14 min. Perfusion with physiological solution was then resumed and a recovery period recorded. The responses were best analysed in terms of changes in beat frequency and reduction in amplitude of the ECG and mechanogram.

The first series of experiments,[10] performed at room temperatures (24 to 33° C.), gave threshold effects at concentrations of the test materials of about 0.001 to 0.01 *M*, but more consistent and dependable responses were obtained at 17 to 19.5° C. A second series of experiments, summarized in TABLE 1, was carried out at 17 to 19.5° C. with threshold responses occurring at slightly higher concentrations.

To be assured that our preparations and recording system were well behaved, acetylcholine was perfused at various concentrations. No significant difference occurred between the speeding of the heart by acetylcholine in our preparations and those of Welsh.[8] FIGURE 4 illustrates the response of the lobster heart to homarine* at 29° C. Perfusion with the test solution began at the arrow and 24 sec. later, in the 0.016 *M* case, the material reached the heart, as indicated by the characteristic gross swing of the ECG record (the upper trace of each pair). This gross change in the ECG trace probably resulted from a modification of the concentration or polarization potentials of the intracannular electrode by the perfusion solution. About 50 sec. after the beginning of perfusion the action of the test material on the ganglion cells slows the heart, and in this case caused extra beats to appear.

Similar double beats were occasionally seen at the higher temperatures in response to pyridine (1.4×10^{-5} *M*) and eserine (1.82×10^{-7} *M*). Since this response was not constantly observed, it may have been more characteristic of particular preparations than of particular drugs. Pyridine frequently caused a prolonged and heightened positive wave on the ECG following the fast negative wave associated with cardiac contraction.

The more common responses for the drugs listed in TABLE 1 were slowing of the beat frequency and reduction of the amplitude of the ECG and mechanogram. No unique effectiveness of homarine or any one of the materials was revealed in these experiments. At 17.0 to 19.5° C. the beat amplitude and frequency were usually reduced for 0.0114 *M* concentration of all materials, with greater reduction or block for 0.14 *M*. In the case of isonicotinic acid, a small increase in activity was seen at 0.00014 *M*, while at 0.0014 *M* the observations were inconclusive. Pyridine and isonicotinic acid methyl betaine (0.14 *M*) reversibly blocked the heart in diastole, and α-picolinic acid (0.14 *M*) reversibly stopped the heart near midposition of the normal contraction. Since there was always a concomitant change in ECG and mechanogram when the heart was altered, one can assume the responses resulted from the action of the compounds on the cardiac ganglion and not on the muscle directly.

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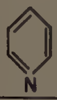
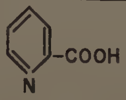
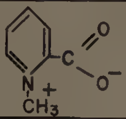
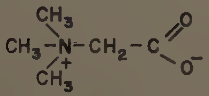
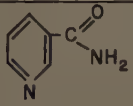
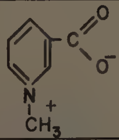
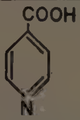
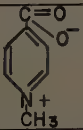
* Homarine for the initial experiments was kindly supplied by J. L. Strominger and H. M. Kalckar.

TABLE 1

SUMMARY OF LOBSTER HEART PERFUSION EXPERIMENTS AT 17–19° C.*

Compound		#	Conc.	Beat Frequency	Beat Amplitude
PYRIDINE		4	0.0014 M	N	N
		11	0.014	D	D
		10	0.14	Stops in diastole	
α-PICOLINIC ACID		10	0.0014	N	N
		12	0.014	D	D
		7	0.14	Stops in mid-position	
HOMARINE		6	0.00116	N	N
		9	0.0116	D	D
		7	0.116	D	D
BETAINE	 $CH_3-\overset{CH_3}{\underset{CH_3}{N^+}}-CH_2-C\overset{O}{\underset{O^-}{}}$	6	0.0014	N	N
		7	0.014	N	N
		6	0.14	D	D
NICOTINAMIDE		1	0.0014	N	N
		1	0.014	D	D
		1	0.14	D	D
TRIGONELLINE		7	0.0014	N	N
		8	0.014	N	N
		7	0.14	D	D
ISONICOTINIC ACID		4	0.00014	I	I
		9	0.0014	?	?
		8	0.014	D	D
		9	0.14	D	D
ISONICOTINIC ACID METHYL BETAINE		2	0.0014	D	D
		2	0.014	D	D
		2	0.14	Stops in diastole	

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* The effects on frequency and amplitude are designated by: N, no change; D, decrease; I, increase; ?, inconclusive; and #, number of observations.

The addition of these compounds to make concentrations of 0.14 *M* increased the osmotic pressure of the perfusate about 14 per cent. Cole[9] observed that increases in osmolarity up to 15 per cent caused no significant changes in heart beat provided the ionic ratios were maintained. Above 15 per cent an irreversible block of the heart in diastole was reported. While our increased molarities approached this limit, the brief exposures to test solutions and the quick response with ready reversibility argue against an osmotic effect.

Since the threshold for these perfusates is very high in contrast to that for AChCl (10^{-9} M), one must conclude from the lobster heart perfusion studies that it is unlikely that homarine and any of these related compounds have a neurohumoral role. Additional indications for a passive role of homarine are found in the investigations of M. J. Keyl *et al.*,[11] whose chromatographic eluent, "homarine peak," did not produce contraction of the frog rectus abdominis muscle, and in the investigations of J. H. Welsh and P. B. Prock,[12] who found that injection of homarine solutions into crabs did not produce the autotomy reflex.

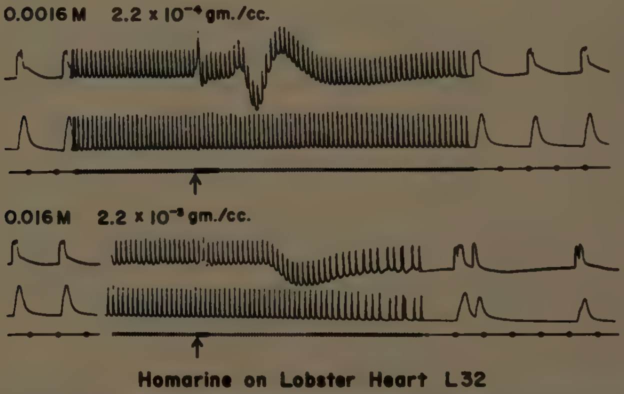

FIGURE 4. Responses of the perfused lobster heart to two concentrations of homarine The ECG is the first and the mechanogram is the second trace of each pair. The time marks are at 1-sec. intervals. The record is speeded (*at right*) to show detail.

Distribution of Homarine in Tissue and Phyla

The distribution studies[13] were undertaken according to the tenets of the comparative biochemists, who feel that determination of the locus of a compound within a biological system may supply clues to its function and structure. Such studies could thus reinforce or negate our original concept: that homarine was of basic importance to functioning nerve and muscle throughout the animal kingdom. Aside from this initial motivation, the study was markedly broadened by the relative ease with which the quantification of homarine could be achieved by means of its UV absorption peak. This broader study not only enabled us to reject our early assumptions but led to important observations concerning the significance of homarine in animal evolution.

For the bulk of the distribution survey, assays were made from UV absorption of aqueous extracts of the tissues investigated. Fresh tissue (2 to 200 mg.) was ground in a bottom-tapered hand homogenizer until the tissue was thoroughly dispersed. These suspensions were centrifuged until clear, the supernatant was poured into quartz absorption cells, and the UV absorption spectra were determined on a spectrophotometer. For all tissues containing a high concentration of homarine, a distinct absorption peak in the vicinity of 272 mμ was always seen. An absorption spectrum for such an aqueous extract of lobster heart is shown in FIGURE 5, curve *A*. To obtain more correct values for the observed absorption curve, the plots were altered by subtracting background, represented by a line drawn from *a* to *b*. This correction resulted in curve *C*. Upon chromatographing the sample on a resin column and then measuring its absorption, curve *B* resulted, and a remarkable agreement with the corrected curve *C* was obtained. Nine corrected measurements of this type made on hearts of *Homarus* gave an average value of 3.1 mg./gm. wet weight as compared to 3.0 mg./gm. for seven measurements of the same tissue on the column. Similar agreement was obtained in three other tissues, which were processed both ways (TABLE 2).

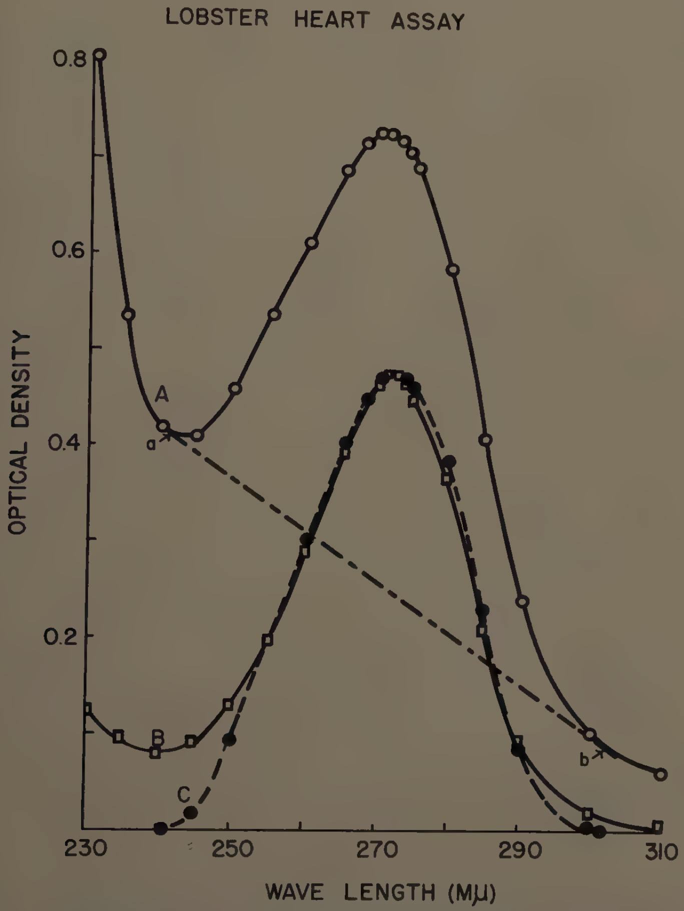

FIGURE 5. Comparison of the crude extract assay method with the chromatographic method. Curve *A* represents an aqueous extract of lobster heart. Curve *C* is obtained from curve *A* by subtracting the background, *ab*. Curve *B* is obtained by chromatographing a sample for curve *A*.

To illustrate the types of absorption curves encountered when using the approximation method, curves for several tissues are plotted in FIGURE 6. *Homarus* claw muscle and *Loligo* giant axon give plots similar to *Homarus* heart (FIGURE 5), with maxima very close to 272 and minima between 240 and 245 mμ. When the maxima of the corrected curves for these and other tissues did not fall between 270 and 275 mμ, it was concluded that homarine was not present unless revealed by another method. *Loligo* blood, *Lymnea* foot muscle, and *Mustelus* muscle gave plots illustrative of tissues not revealing homarine, at least not by the approximation method.

TABLE 2
HOMARINE DISTRIBUTION IN TISSUES
(mg./gm. wet weight)

	Loligo		*Homarus*		*Limulus*	
	#	(mg./gm.)	#	(mg./gm.)	#	(mg./gm.)
Liver or hepatopancreas	4	7.4	2	2.4	1	6.6
			1	2.3*		
			2	1.2†		
Gill	4	5.1	1	2.3	2	2.0
Mesentery	3	2.7				
Skin	2	2.0				
Stomach	3	1.9			2	3.5
Blood	1	0.028*	2	0.050†	1	0.07
					3	0.185†
Urine			2	0.034		
			2	0.049†		
Nerve						
Cerebral ganglia	4	7.6	2	2.9	2	9.3
			3	2.6†		
Peripheral nerve	12	1.4	2	2.1	2	7.7
Ventral n. cord					1	10.3
Optic nerve					4	9.0
					3	9.9†
Giant axon	7	1.7				
Muscle						
Systemic heart	4	1.3	9	3.1	2	7.6
			8	3.0*		
			3	2.1†		
Branchial heart	5	4.4				
Abdominal			1	2.2	1	7.7
			1	2.1*		
Mantle	5	1.2				
	1	1.1*				
Chela			1	4.4		
Siphon	4	1.3				
Leg					2	6.8

* Extracted on resin.
† Extracted on paper.

Tissues in which a low concentration of homarine was found presented greater problems of analysis since the homarine absorption peak was frequently obscured by extremely high background and absorption peaks of other materials. Obviously in such cases the background correction method was not applicable. For instance, in order to obtain a measurement of the homarine concentration for the blood of squid, it was necessary to abandon the above approximation method because of the high absorption of blood proteins (FIGURE 6). In such cases it was necessary to resort to column or paper chromatography, using the methods described earlier; for these measurements the column was smaller (12 cm. long), and was used with appropriately smaller quantities of materials.

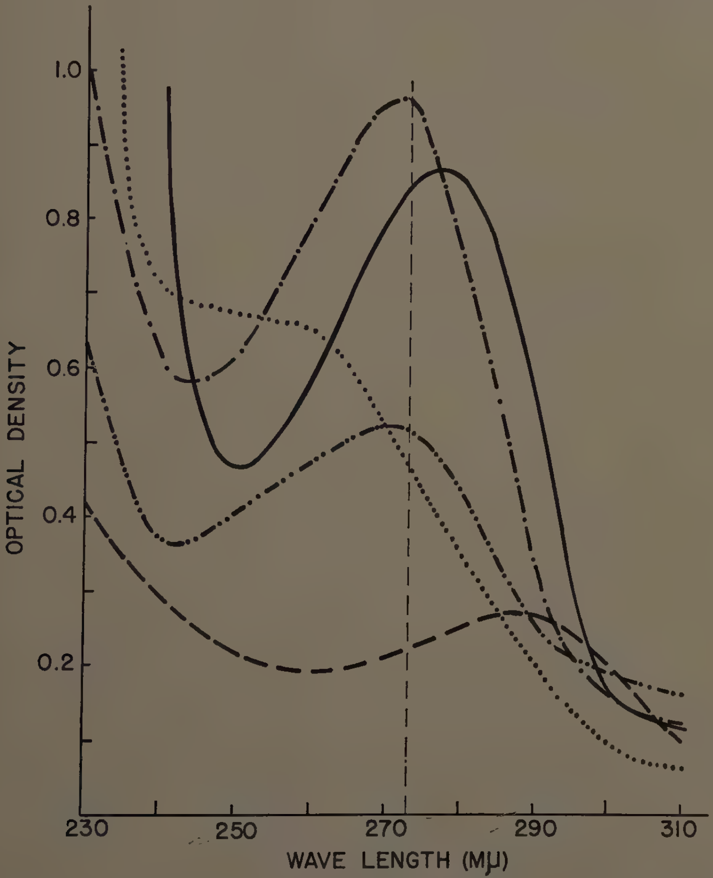

FIGURE 6. Absorption spectra of crude aqueous extracts on various tissues. Key: ——— *Loligo* blood; – – – – – *Lymnea* foot; –·–·–·– *Homarus* claw; –··–··– *Loligo* giant axon; and ······· *Mustelus* muscle.

Usually the muscle, nerve, gill, and whole animal were processed for assay, but in the cases of *Loligo*, *Homarus*, and *Limulus* a wide survey was made. TABLE 2 summarizes our findings for these species. All assays were made by the approximation method, except in the cases indicated by the symbols, for which samples were run on the small column (*) or on paper (†). The table reveals a wide distribution of homarine within the tissues of a given species but, in general, *Loligo* has more than *Homarus*, and *Limulus* has more than

Loligo. Nerve and muscle often showed very high concentrations, such as 10.3 mg./gm. wet weight for ventral nerve cord of *Limulus* and 7.6 mg./gm. for cerebral ganglia of *Loligo*. Glandular tissue such as hepatopancreas or gonads contained concentrations nearly as great, while the skin, mesentery, and stomach contained less. The lowest concentrations appeared in the blood and urine, where assay by paper and resin column was necessary.

Quite unexpectedly, it was discovered that the giant axon of *Loligo* displays an unequal distribution along its length. FIGURE 7 illustrates the results of an experiment in which a giant axon was divided into four sections and the concentration determined as a function of the distance from the cell body.

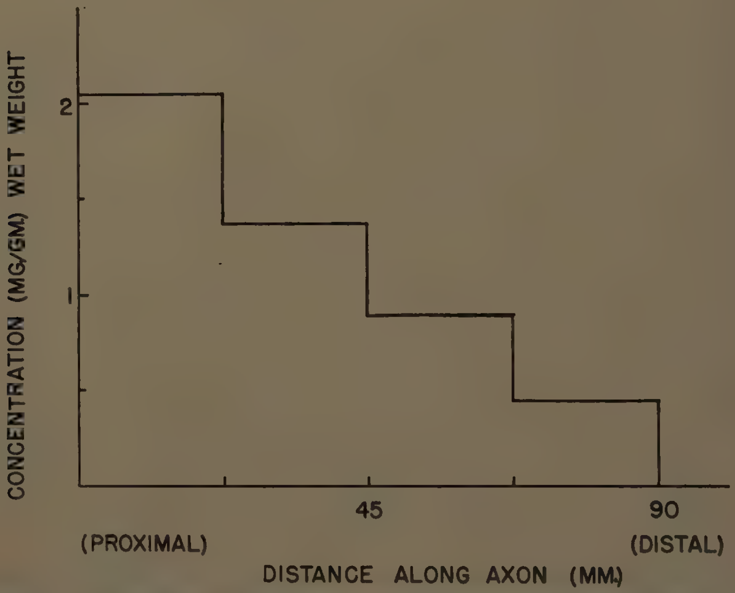

FIGURE 7. Concentration of homarine in the giant axon of the squid (*Loligo*), as a function of the distance from the cell soma.

This relation was found in 8 of 13 axons assayed, whereas it could not be established in 6 experiments on the optic nerve of *Limulus*.

Eleven species of sea plants collected from the Cape Cod, Mass., area were examined by paper chromatography without finding evidence for the presence of homarine.

The breadth of our survey can be seen from TABLE 3, in which the phyla and species investigated are tabulated with the corresponding homarine concentration. Representation of ranges of concentrations by + signs was chosen because of the crudeness of the assay method and because a far better perspective of the relative concentrations is gained in this way. Concentrations greater than 6.0 mg./gm. wet weight are represented as ++++, 3.0 to 6.0 as +++, 1.0 to 3.0 as ++, and 0.01 to 1.0 as +. "NF" indicates that no homarine was found by our methods and "?" indicates that homarine might be present in very small amounts.

The two most striking findings from this distribution study are the absence of homarine in fresh-water invertebrate and vertebrate forms. The more complicated marine invertebrates tend to have a high concentration of homarine. However, it is interesting that this is not a consistent finding, for in the case of some crustaceans it was not unusual to detect very little or possibly no homarine, for example, in *Pagurus* and *Uca*.

TABLE 3

THE DISTRIBUTION OF HOMARINE BY PHYLA

Phylum	Species	Whole animal	Heart	Skeletal muscle	Ganglia	Peripheral nerve	Gill
Porifera	*Scypha* (sponge)	+					
Coelenterata*	*Metridium* (anemone)	+					
Ctenophora	*Mnemiopsis* (sea walnut)	+					
Annelida	*Nereis* (worm)	++					
Mollusca*	*Anodonta*† (clam)		NF				NF
	Pecten (scallop)		+	+			+
	Venus (clam)		+	+	++		++
	Amnicola† (snail)	NF					
	Lymnea† (snail)	NF					
	Nassa (snail)	++					
	Loligo (squid)		++	++	++++	++	+++
	Busycon (whelk)		+++	+++	++++	++++	+++
Arthropoda*	*Carcinus* (green crab)		++	++	++	++	++
	Pagurus (hermit crab)	?					
	Uca (fiddler crab)	?					
	Cambarus† (crayfish)		NF	NF	NF	NF	NF
	Homarus (lobster)		++	++	++	++	++
	Limulus (king crab)		++++	++++	++++	++++	++
	Musca (house fly)	NF					
Echinodermata*	*Asterias* (star fish)	?					
	Thyone (sea cucumber)			NF			
	Arbacia (sea urchin)	+					
Urochordata	*Molgula* (sea squirt)	+					
Vertebrata	*Mustelus* (dog fish)		?	NF	NF	NF	?
	Prionotus (sea robin)		NF	NF	NF	?	?
	Felis (cat)					NF	
	Rana (frog)		NF	NF		NF	

Key: ++++, more than 6.0 mg./gm.; +++, 3.0 to 6.0 mg./gm.; ++, 1.0 to 3.0 mg./gm.; +, 0.01 to 1.0 mg./gm.; ?, suggested, but not certain; and NF, not found.

* Reported by other investigators.

† Fresh-water forms.

Discussion

The widespread presence of homarine in invertebrate tissues reveals a role more general than we had conceived for it in excitable tissues such as nerve and muscle. Its ability to diffuse from cells despite its quaternary form, and its low concentration in blood and urine indicate that it may be a metabolic end product (see discussion by A. H. Mehler[14]). The unequal distribution along the squid giant axon can be interpreted in accord with this view. Should homarine be produced primarily in the soma, the metabolic center of the nerve cell, it might be lost through the axonal membrane after diffusing or being pumped from the soma. Greater amounts would be lost centrally with less remaining to move peripherally. Attempts to support this theory by relating changes in concentration with changes in axonal volume or surface area and by determining axoplasm-sheath:homarine ratios were unsuccessful. Thus until further investigations are available, it is well to remember that other explanations of the homarine gradient are equally tenable.

The most important finding is the absence of homarine in the fresh-water invertebrate forms.[13] From this observation one can postulate that homarine is lost from the metabolic pathways with the evolution to fresh-water species. A natural consequence of the loss of this unbound cellular constituent would be a small but desirable reduction in the cellular osmotic pressure for fresh-water life, if one assumes that in a biochemical evolution the material is not replaced in the metabolic pathways by a similar compound. A possible alternate change could be an increased cellular permeability to homarine in fresh-water forms; thus the material would be allowed to escape very rapidly without accumulating to readily detectable levels. That such osmotic effects may be considered is evident for the *Limulus* optic nerve and ventral nerve cord, in which homarine may produce up to 7 per cent of the total cellular osmotic pressure. These effects may be even greater if they are shown to extend to other nitrogenous bases, as suggested by Welsh and Prock.[12]

A possible corollary of these interpretations would be the expectation of finding a low or markedly variable concentration of homarine in estuarine forms. *Pagurus* qualifies in this respect and does contain a barely detectable amount of homarine (less than 0.1 mg./gm.). *Carcinus* shows a high degree of variation, although its concentration is not especially low. However, in opposition to the foregoing suggestion is the finding that *Uca*, which has poor osmoregulatory capabilities, contains only small amounts of homarine as does *Pagurus*.

More important implications of the distribution study can be appreciated upon examining the concentrations as they vary from phylum to phylum. Although homarine has been reported in various phyla (Coelenterata,[12,15] Mollusca,[11,16] Arthropoda,[4,17] and Echinodermata[4,6]), a more extensive study such as this can be uniquely rewarding despite its seemingly tedious nature. This point is emphasized upon mapping our results on the phylogenetic tree, as in FIGURE 8. Here the concentrations represent the maxima obtained within a phylum or class and have the same ranges as those listed in TABLE 3.

It is evident that homarine is distributed in a rather systematic manner along this particular phylogenetic tree, increasing in concentration with evolution from the simpler to more complex forms. Greatest concentrations are reached in the large marine invertebrates such as *Loligo*, *Busycon*, and *Limulus*. Increasing size, with the associated decreasing surface area:volume ratio, might be a factor if the circulatory and excretory mechanisms were not adequately efficient in the larger species. That this is not the case is indicated by the very low urine and blood levels of homarine. Our attempts to demonstrate a possible relationship between size and concentration within a species were unsuccessful. Therefore it seems far more likely that the increased concentrations in these complex invertebrate forms are more directly related to cellular activity than to systemic organization. A direct dependence on cellular activity is also consistent with the osmoregulatory role discussed previously.

In the branch of the phylogenetic tree leading to the vertebrates, a low concentration in echinoderms and a urochordate is found, with none appearing

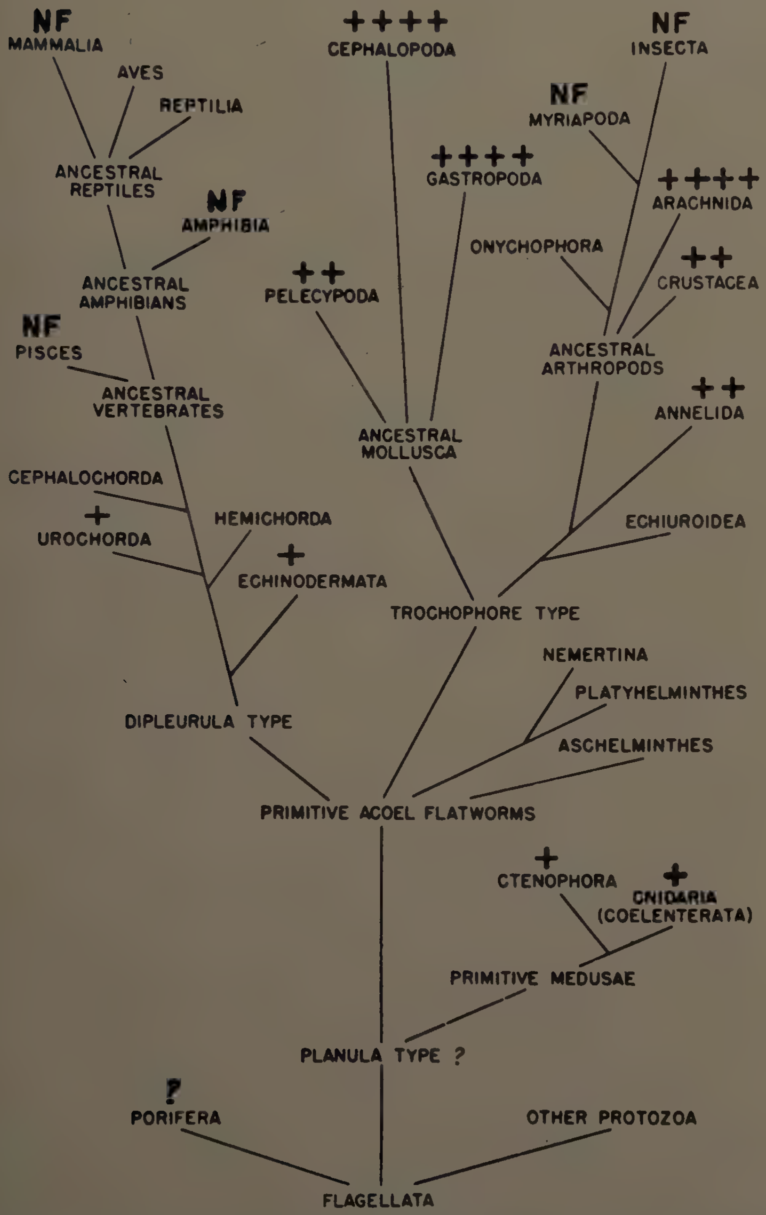

FIGURE 8. Phylogenetic distribution of homarine. Key: ++++, above 6 mg./gm. wet weight; +++, 3 to 6 mg./gm.; ++, 1 to 3 mg./gm.; +, 0.01 to 1 mg./gm.; ?, suggested but not certain; and NF, not found. Homarine was not present in fresh-water forms. *Limulus* was the species used for class Arachnida. Adapted from Hyman; reproduced by permission from *Comparative Animal Physiology*.[19]

in the vertebrates. This finding is in agreement with the postulated origin of the vertebrates from the echinoderms, a concept derived from morphological evidence and receiving support from the distribution of phosphagen.[18] The absence of homarine in fresh-water forms is consistent with the view that vertebrates rose from a fresh-water environment. At the same time, however, it argues more for a role in the evolution of all fresh-water forms rather than of the vertebrate forms per se.

As stimulating as speculation may be, facts, as truth, are much more revered. Further facts about concentration determinants for homarine and similar quaternary ammonium compounds, particularly in the phyla Annelida, Echinodermata, and Protochordata, are clearly needed.

Acknowledgments

Among our many helpful associates we particularly thank David Elwyn and Victor Whittaker. The capable technical assistance of Eva Hauptmann and Doris Blinks is very much appreciated.

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PHARMACOLOGICALLY ACTIVE AMINES AND THEIR BIOGENESIS IN THE OCTOPUS*

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Introduction

The cephalopod octopods and squids have attracted the attention of biologists since the time of Aristotle.[3] Their "salivary" or "venom" glands, whose full function still is unknown, have been studied since the time of Paul Bert in 1867.[4]

General reviews of the glands are available in which their contents, biochemistry, and function are discussed.[5-13] Their general description, species differences, morphology, anatomy, histology, and histochemistry have been well documented.[9-11,14-28] Methods for cannulating the ducts from the glands have been developed, first by Krause[29] in 1897; since then this technique, as well as stimulation of the nerve to the isolated, perfused glands has been used by several investigators who studied their physiology, pharmacology, and biochemistry.[6,7,10,12,30-41]

The physical, chemical, and digestive properties of the gland secretion have been examined by some of the authors quoted above, as well as by many others.[4,7,25,29,42-53] It is generally believed that the posterior glands have little or no proteolytic enzymes, amylases, or lipases, but may contain a hyaluronidase.

The venomous properties of the posterior gland secretion gained early attention. For example, Jousset de Bellesme[50] described the crab paralysis effect of the secretion in 1879. There are more than 40 references on the "venom" in the literature. The subject is reviewed elsewhere in this monograph by Ghiretti, who points out that some of the earlier investigators thought the venom was chiefly tyramine ("Tya"), although Briot and Livon, in 1905 and 1906, believed it was proteinaceous. It remained for Ghiretti to prove this by isolating and purifying the active material in 1959.

Other enzymes found in the gland include amine oxidases[54-56] and flavinic enzymes.[57] Because Tya and histamine ("Hm") are present (*vide infra*), it was first postulated by Bottazzi in 1921[58] and later by Bacq and Ghiretti[7] that tyrosine ("Tyo") and histidine ("Hd") decarboxylases may be present, although this was not experimentally verified until the present work.[1,2]

Other pharmacologically and physiologically active substances found in the glands include the guanidine bases, octopine[59] and agmatine,[99] acetylcholine,[30,32,60] noradrenaline ("NA"),[61] and possibly even 11-hydroxysteroids.[34]

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Preliminary reports on this work have appeared in the *Proceedings of the Western Pharmacology Society*[1] and in *Federation Proceedings*.[2]

The latter observation, coupled with studies of the active amine content, believed by some to be hormones of internal as well as external secretion, led Bacq and Ghiretti[34] to compare the posterior salivary glands to the adrenal glands of vertebrates.

Polyphenols, phenolamines, and indoleamines have been suspected as endogenous substances of these glands since 1909, from histochemical evidence, color reactions, and pharmacological assays,[6,7,17,18,21-24,31,33,34,62-73] particularly Tya, which was first isolated by Henze in 1905,[74-76] and the role and function of which has been studied and speculated upon by many investigators.[6,7,8,12,33,34,38-40,42,47,48,58,69,70,76-82] Meta-tyramine ("*m*-Tya") also has been invoked.[7]

Serotonin (5-hydroxytryptamine, enteramine, "5-HT") was suspected as an endogenous constituent of the posterior glands by Erspamer as long ago as 1948; he subsequently isolated it from the glands and showed it to be identical to authentic material obtained by synthesis. Its function has been postulated to be a constituent of the "venom," a neurohumoral transmitter, and an endocrine, determining, among other things, the nervous control of the melanophore system.[8,64,66,67,77,78,82-92] Its isolation from the glands was a heroic task, requiring the glands from 30,000 specimens of *Octopus vulgaris*.

Octopamine (*p*-hydroxyphenylethanolamine, "Popea") also was found in the glands,[47,65,77-80,82,93] and may be the precursor of the NA present.[61] It is interesting that Popea recently has been demonstrated as a normal constituent of mammalian urine[94] and is present in brain.

Hm was suspected as a normal constituent of the glands by Bottazzi as early as 1916,[42,58,95,96] and since then has been definitely identified;[6-8,41,78] it is postulated to arise from Hd by Hd decarboxylase action,[7] although this was not proven.

Of the amino acids that are possible precursors of some of the amines present, Tyo,[8,47,76] tryptophan ("TP"),[8] and Hd[8] have been demonstrated. However Erspamer[8] could not find *p*-hydroxyphenylserine ("Pops"), the precursor of Popea.

A precursor of NA, 3,4-dihydroxyphenylserine ("Dops"), has not been sought, nor have 3,4-dihydroxyphenylalanine ("Dopa"), 3,4-dihydroxyphenylethylamine (dopamine, "OHT"), and *m*-hydroxyphenylalanine (*m*-tyrosine, "*m*-Tyo"). However, *m*-Tya is thought to be present.[7]

The present study describes some preliminary work on the endogenous amines and amino acid decarboxylases presumably responsible for their formation in the posterior salivary glands of two species of octopods.

Methods

Tissue preparation. The salivary glands of the large north Pacific octopus, *Octopus apollyon*,[14,28] were obtained from a commercial octopus fisherman at Puget Sound, Washington, who kept the glands in glass containers on wet ice during butchering, after which they were transferred to sealed plastic containers on dry ice and shipped by air on dry ice in Styrofoam-insulated containers. The tissues were received within 24 hours from time of removal and were processed at once. A specimen of *O. apollyon* is illustrated in FIGURE 1.

Live specimens of the smaller local species *O. bimaculatus* were obtained from a marine biological supplier and kept in fresh sea water aquaria until sacrificed, usually within 5 to 6 hours, to ensure fresh material. They were killed by destruction of the brain, after which the arms were removed to facilitate extirpation of the glands. The glands were weighed, rinsed, and homogenized in a Virtis 45,000 rpm homogenizer with two parts of glass-distilled water, or with 0.1 *M* phosphate buffer of the required *p*H.

FIGURE 1. A specimen of *Octopus apollyon.*

Protein fractionation procedures usually were carried out with glands of *O. apollyon* because of their large size (8 to 10 gm. each) and the large numbers available (FIGURES 2 and 3). Fractionation was accomplished using saturated ammonium sulfate. For example, approximately 300 gm. of frozen glands were thawed and homogenized with 600 ml. of iced distilled water. To the homogenate at 0° C. was added neutral saturated ammonium sulfate at 0° C. with rapid stirring until the desired percentage saturation was attained. The precipitate was allowed to form at 0° C. for 30 min., after which the mixture was centrifuged in a refrigerated Servall centrifuge at 0° C. and 35,000 g for approximately 30 min. The supernatant fluid was then decanted off for further fractionation. The precipitate was taken up in a minimum volume of distilled water or buffer, and dialyzed in water or buffer at 2 to 4° C. The

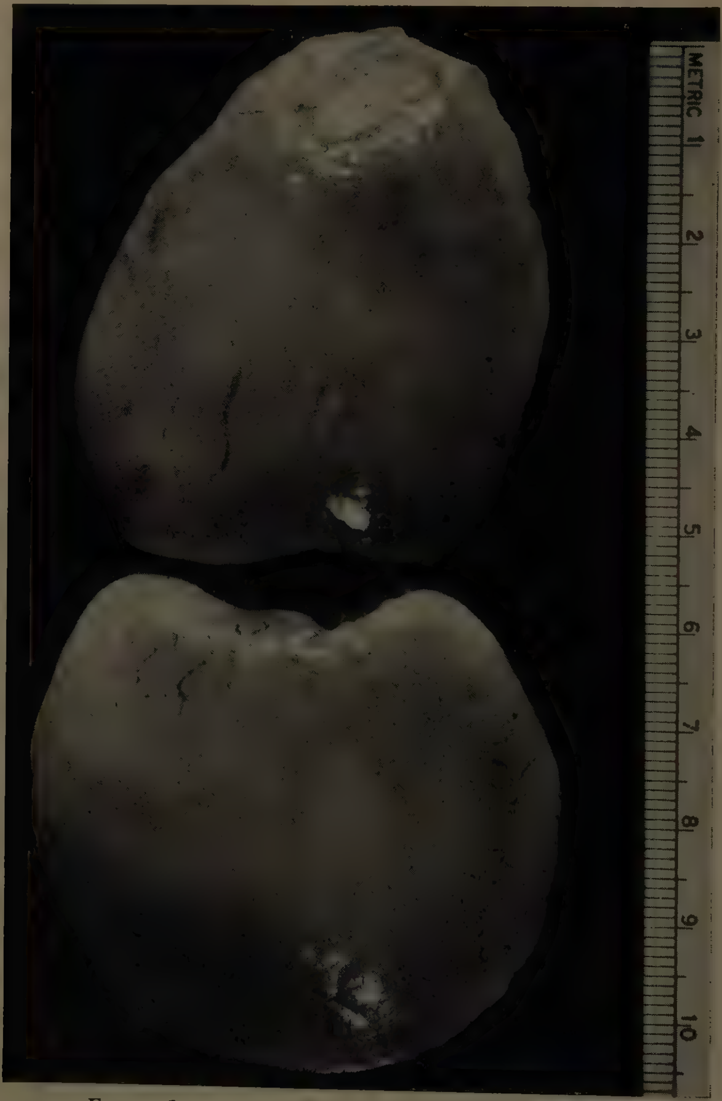

FIGURE 2. Posterior salivary glands of *Octopus apollyon*.

fractionation, especially the centrifugation and dialysis times, are fairly critical for maximum enzyme activity. To minimize this time, a continuous-flow centrifuge head on the Servall ("Szent-Gyorgyi-Blum" head) is desirable, as is a rapid dialysis apparatus. To minimize dialysis time, a special apparatus was constructed that is very efficient. Essentially it is a large (4 inches in diameter × 12 inches long) transparent plastic pipe, which is rotated slowly end over end at an approximate rate of 5 rpm on its center axis by a geared motor.

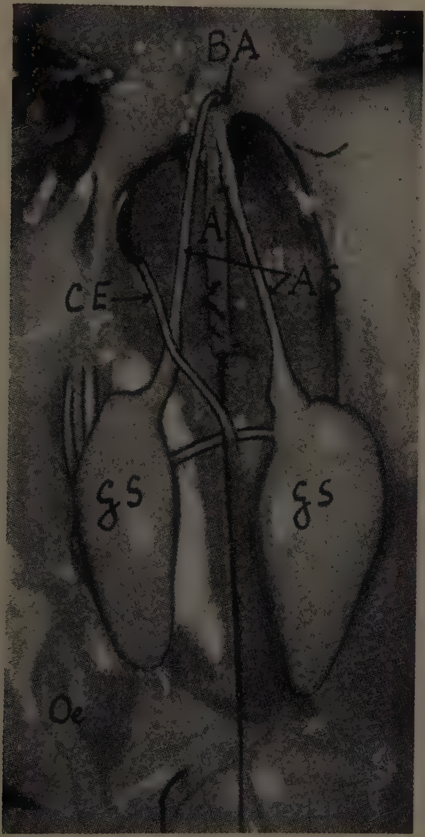

FIGURE 3. A preparation of the salivary glands of *Octopus macropus* before the ligature of the branches of the aorta. Key: Oe, the esophagus, sectioned and tied off in back; A, the ligated and sectioned aorta, with the black threads indicating the ligated branches; GS, the posterior salivary glands with their excretory canal; CE, the ligated common excretory canal; AS, the posterior salivary arteries; and BA, the bifurcation of the dorsal aorta and the ligatures of the two pharyngeal arteries.

Reproduced by permission from *Archives internationales de physiologie.*[31]

The ends of the plastic pipe are threaded over plastic end plates. Removable plastic rods, eight in all, fit inside the pipe and are held in place between the two plates in recesses. Dialysis tubing is tied on or around these rods. Water at 0° C. enters at the axis on one side of the pipe through a plastic joint and leaves by the other, the flow being controlled by a needle-valve stopcock. A bubble of air in the dialyzer and a bubble in the dialysis tubing give efficient stirring during dialysis. At intervals of 1 hour or less, 1 ml. of solution is withdrawn from each dialysis bag and tested for ammonium ion with Nessler's solution. When no color develops (usually after about 8 hours), the dialysis bags are removed and the contents used for Warburg or radiochemical studies on the enzyme content.

If gland tissue is to be studied for detection of endogenous amines instead of for enzyme activity, timing is not critical, and the tissue can be stored deep-frozen until needed. It then is homogenized in acetone or in acid alcohol; the supernatant fluid is lyophilized or evaporated at reduced pressure and temperature, is passed through alumina, Dowex CG-50, or cotton-acid-succinate chromatographic columns, and the eluates and washings are spotted on chromatographic paper and allowed to develop with appropriate solvent systems, along with appropriate authentic samples, for comparison of the R_fs.

Manometric methods. Enzyme activity initially was followed by manometric methods with conventional Warburg apparatus, using conical vessels with two side arms. The enzyme was added to the main compartment as a homogenate, dialyzed ammonium sulfate fraction, or solution of lyophilized powder, as indicated below in each table. Then 1.5 to 2.5 ml. of 0.1 *M* phosphate buffer, *p*H as indicated, were added to the main compartment, as well as 40 μg. of pyridoxal-5-phosphate ("B_6PO_4," "PP"). One side arm usually contained 10 μmole of substrate; the other side arm contained 0.2 ml. of 3 *N* H_2SO_4 or $HClO_4$. When amine end products were to be determined, 100 μg. of aminoguanidine and/or 100 μg. of isobutylisonicotinic hydrazide were added to the main compartment to inhibit diamine or monoamine oxidase. The flasks were deaerated with nitrogen and equilibrated to 37° C. for about 10 min. After a prereading, the substrate was tipped in from the side arm and the evolved CO_2 was recorded every 15 min. for 1 to 3 hours. At the end of the desired time the acid was tipped into the reaction chamber to liberate trapped CO_2 and a final reading was taken 20 min. later.

Paper chromatography. One-dimensional paper chromatography was employed to determine the endogenous amines and amino acids present in the glands and the amines formed, and to correlate and identify radioactive substances separated by column chromatography and counted in a flow counter. The usual descending type of all-glass chromatography jar, troughs, and rods was used in most procedures. Whatman No. 1 paper was used unless otherwise noted. Several solvent systems were used; most frequently, the solvents were isopropanol-acetic acid-water (70:5:25 v/v/v); butanol saturated with 1 *N* HCl; and phenol-1 *N* HCl-SO_2 (sat.). Several sprays were used to develop the spots, applied singly or in succession. The most effective sprays were diazotized sulfanilic acid; potassium ferricyanide-ferric sulfate-HCl; *N*,2,6-trichloro-*p*-benzoquinoneimine, 0.1 per cent w/v in anhydrous ethanol or freshly-prepared methanol, followed by 2 per cent borate buffer, *p*H 9.5; and ninhydrin, 0.5 per cent in 90:10 v/v pyridine-acetic acid.

Two-dimensional paper chromatography is being employed in experiments in progress, to further verify these findings.

Radioactive methods. Hd decarboxylase activity was estimated by using

L-Hd-2-(ring)-C^{14} as substrate. Fresh homogenates of octopus glands were incubated for three hours in the Dubnoff metabolic incubator at 37° C. with the substrate, 0.1 *M* phosphate buffer of varying *p*H, 40 μg. of B_6PO_4, and 100 μg. of aminoguanidine to inhibit any diamine oxidase present. One drop of toluene was added to inhibit bacterial growth. After incubation, blood agar plates were streaked with a loop of incubate from each flask and, if bacterial growth appeared, the experiment was discarded.

The enzymes were killed and proteins precipitated by adding two drops of concentrated $HClO_4$; the precipitate was then filtered or centrifuged off and the precipitate was washed with 0.2 *N* $HClO_4$. The supernatant fluid and washes were then treated by a modification of the isotope dilution method of Schayer *et al.*,[97] which consists of extraction of Hm into butanol with the aid of sodium sulfate and washing the butanol layer with alkaline Hd solutions to remove residual C^{14}-Hd. For example, 37 mg. of unlabelled L-Hd was used as a carrier, and 66 mg. Hm-HCl as the diluent. The Hm was re-extracted into aqueous HCl, dried, and converted to the dipicrate by the original method of Schayer,[98] using 180 mg. of picric acid. Successive recrystallizations of the Hm picrate from 50 per cent alcohol with Norite charcoal yielded constant activity after four such recrystallizations, and thus a pure end product. This was counted in an automatic planchet changer-flow counter at infinite thickness.

The gravimetric yields were recorded and, if they were found to be below values of the thickness plateau, the experiment was rejected.

Radioactive experiments were performed using L-Dopa-3-C^{14}, 2-C^{14}-DL-*erythro*-Dops,[100] DL-5-Hydroxytryptophan-2-C^{14} ("5-HTP"), and L-Tyo-C^{14} (uniformly labelled) as substrates, using essentially the same incubation mixtures as for Hd, except that 100 μg isobutylisonicotinyl hydrazide were added to inhibit any monoamine oxidase present. After incubation, the reaction was stopped and the proteins precipitated with two drops of concentrated $HClO_4$ to give a final $HClO_4$ concentration of 0.4 *N*. The precipitate was washed with 0.2 *N* $HClO_4$ and the supernatant fluid cooled, and the *p*H was brought to 4 with 5 *N* K_2CO_3 slowly in the cold. The precipitate of $KClO_4$ was washed and discarded. The supernatant containing the amines corresponding to the above mentioned substrates then was adjusted to *p*H 6.1, using ammonium acetate and ammonia, and then passed slowly through a 1 × 20 cm. Dowex CG-50 ion exchange column. The column was eluted on an automatic fraction collector with 0.4 *M* ammonium acetate at *p*H 5 at a rate of 3 to 8 ml. per hour, and 1 to 3 ml. per tube. A portion of the collected solution in each tube was analyzed for absorption at 279 mμ in the Beckman DU spectrophotometer, and the optical density (O.D.) recorded and graphed. Tubes containing substances of high O.D. at 279 mμ were pooled according to the positions of their peaks and the solutions were lyophilized. The lyophilized powders were taken up in small volumes of alcohol or water, spotted on Whatman No. 1 paper and developed by the descending technique in one of the solvent systems mentioned in the section on paper chromatography. After development, the paper was dried and 2-cm.-wide strips were cut down the length of the paper, corresponding to the path of the unknowns. The strips then were cut across at 1-cm. intervals. Numbers corresponding to each 1 ×

2 cm. strip were recorded on the remaining chromatogram, which contained the developed unknowns and suspected authentic standards. The 1 × 2 cm. strips were counted in the automatic planchet changer and the counts per minute (cpm) plotted against strip number. The strips then were reassembled onto the original chromatogram and the reconstituted paper sprayed with appropriate reagents to see if the radioactivity peaks corresponded with the R_f values of known identity.

Results and Discussion

After reviewing the results of others, discussed in the introduction, on the amines and amino acids found in the glands, preliminary investigations were initiated on the amino acid decarboxylases most likely to be present.

Previous investigators have reported species differences in the amine content of the posterior salivary glands of cephalopods. Therefore it was thought pertinent to the present studies to survey the glands for endogenously occurring amines. FIGURE 4 shows a chromatogram of an acid-ethanol and an acetone extract of *O. apollyon* posterior glands, run on Whatman No. 1 paper by the descending technique, developed by isopropanol-acetic acid-water (70:5:25 v/v/v), and sprayed with diazotized sulfanilic acid. The ethanol extract ("S_{EtOH}") shows the presence of Hd, Hm, possibly Tyo, 5-HT, and Popea. FIGURE 5 shows a chromatogram of a similar extract, developed in phenol-1 *N* HCl-SO_2 (85:15:sat'd), and sprayed with the potassium ferricyanide-ferric sulfate reagent. This solvent separates catechols possibly present in the mixture. The sample (*arrow*) shows a definite spot at the R_f of authentic OHT, and two other distinct spots at R_fs corresponding to NA and adrenaline ("Epi"). The possibility of the presence of dopacetic ("DOPAA") and metanephrine ("MeONA") has not been ruled out. FIGURE 6 shows the same extract developed in *n*-butanol saturated with 1 *N* HCl and sprayed with diazotized sulfanilic acid and with ferricyanide-ferric sulfate. The R_fs indicate the presence of the same amines as before, but the samples ("S_{AI}" and "S_{Ac}") also show an additional R_f above Popea, which has been identified by others as a decomposition product of 5-HT but not of 5-hydroxyindoleacetic acid ("5-HIAA").

FIGURE 7 shows the endogenous amines thus far found in *O. bimaculatus* glands. Traces of Hd and Hm, a trace of 5-HT (shown by ultraviolet absorption only), and a small but definite amount of Popea were indicated. The consistently appearing R_fs corresponding to authentic *m*-Tya are more probably due to Tya. Consistently present also were R_fs corresponding to authentic 5-HIAA. The decarboxylations thus far sought are shown in FIGURE 8.

A summary of the Warburg manometric results is shown in TABLE 1.

Using a 1:1 whole homogenate of the fresh-frozen posterior salivary glands of *O. apollyon*, a small but definite decarboxylation may be noted with each substrate. A B_6PO_4 requirement was noted in many but not all instances, and even showed slight inhibition in other instances. This may be due to varying amounts of endogenous cofactor.

The low activities shown in TABLE 1 suggested the possibility of the presence of an endogenous inhibitor, released by homogenization. In order to test this concept, lyophilized hog kidney extract supernatant fluid was used as a source

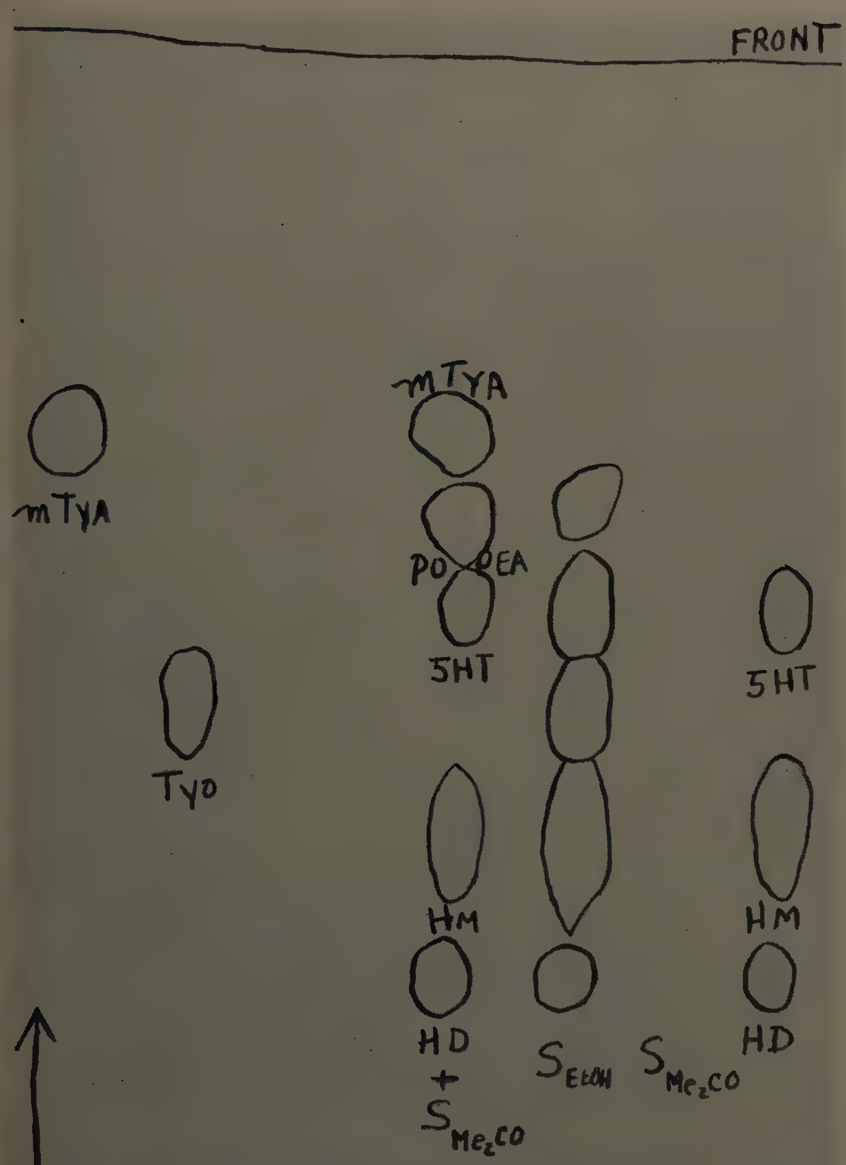

FIGURE 4. Paper chromatogram of 226 gm. of posterior salivary glands of *Octopus apollyon* for endogenous amines. Acid-ethanol and acetone extracts of the glands were run on Whatman No. 1 paper, developed by isopropanol-acetic acid-water, and sprayed with diazotized sulfanilic acid. The ethanol extract (S_{EtOH}) shows the presence of Hd, Hm, possibly Tyo, 5-HT, and Popea.

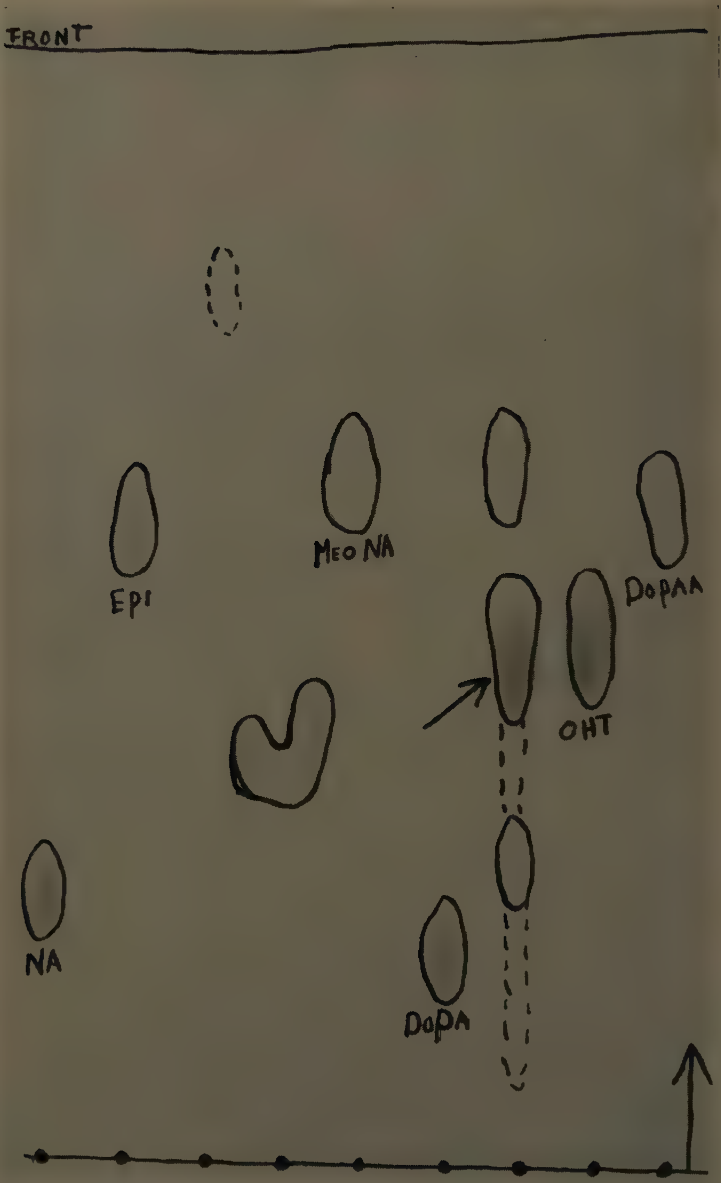

FIGURE 5. Chromatogram of an extract similar to that in FIGURE 4, developed in phenol-1 *N* HCl-SO_2, and sprayed with the potassium ferricyanide-ferric sulfate reagent. The sample (*arrow*) shows a definite spot at the R_f of authentic OHT, and two other distinct spots at R_fs corresponding to NA and adrenaline (Epi).

of Dopa and 5-HTP decarboxylase. The salivary gland homogenate was added to the Dopa and 5-HTP decarboxylases and, as TABLE 2 shows, there was a 25 to 30 per cent inhibition. This inhibition still occurred after 6 hours of dialysis of the gland homogenate and was only slightly reduced by lyophilization of the homogenate. Boiled extract of homogenate was inactive, as were the solids remaining after boiling. Thus the inhibition would seem to be due to a heat-labile, nondialyzable factor.

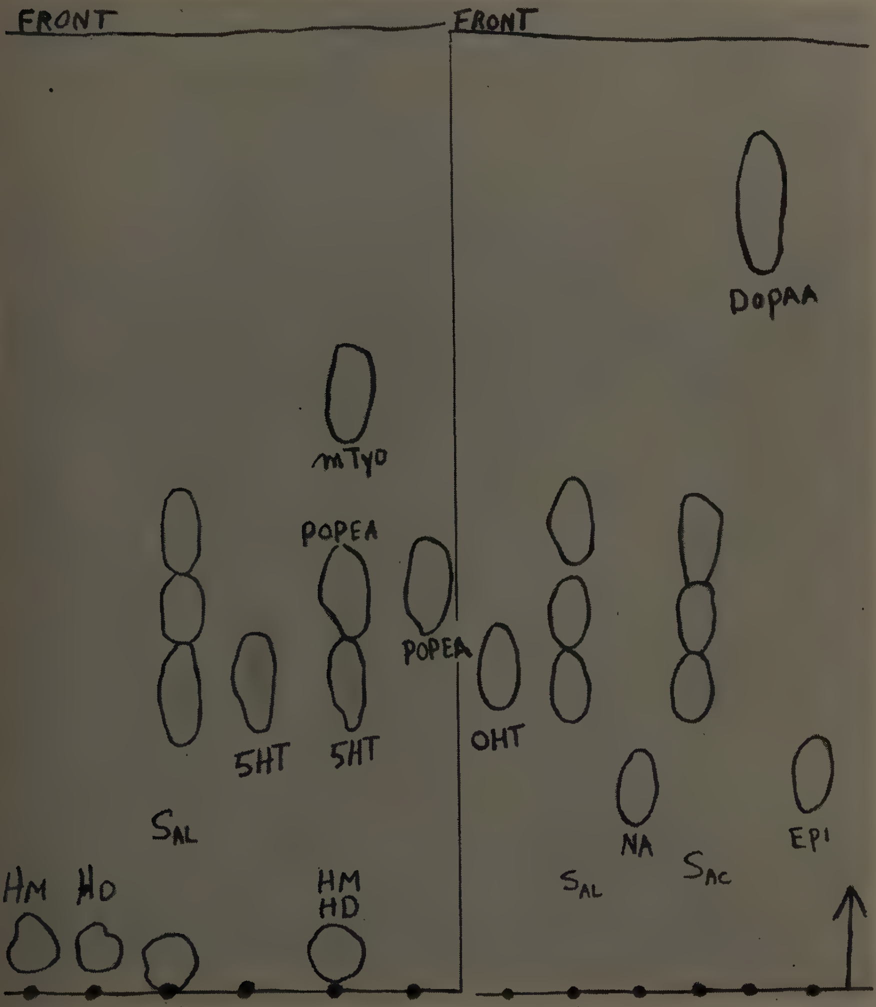

FIGURE 6. The same extract used in FIGURES 4 and 5 is shown here after being developed in *n*-butanol saturated with 1 *N* HCl. The extract was sprayed with diazotized sulfanilic acid (*left*) and with ferricyanide-ferric sulfate (*right*). The samples S_{Al} and S_{Ac} show an additional R_f above Popea that is said to be a decomposition product of 5-HT, but not of 5-hydroxyindoleacetic acid (5-HIAA).

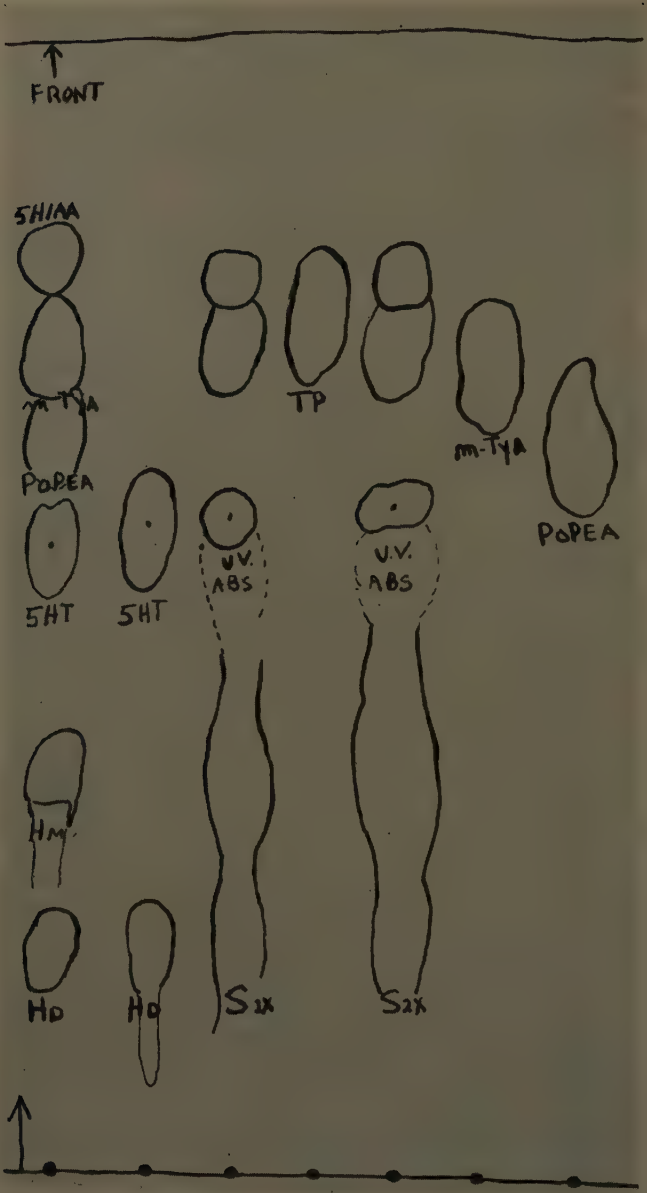

FIGURE 7. The endogenous amines thus far found in *O. bimaculatus* glands. There were traces of Hd and Hm, a trace of 5-HT (shown by ultraviolet absorption only), and a small but definite amount of Popea. The consistently appearing R_fs corresponding to *m*-Tya more probably are representative of Tya.

In order to remove inhibition, an attempt was made to purify the enzymes by ammonium sulfate fractionation. The proteins that precipitated at 30 per cent saturation were discarded. The 30 to 47 per cent fraction was used as an enzyme source for 5-HTP decarboxylation. TABLE 3 shows the inhibitory effect of incomplete removal of ammonium sulfate because of insufficient dialysis, illustrating the necessity of testing for complete dialysis by Nessler's test.

TABLE 1

MANOMETRIC ESTIMATION OF AMINO ACID DECARBOXYLASES IN POSTERIOR SALIVARY GLAND OF *OCTOPUS APOLLYON*

	Expt. No.															
	1*		2		3†		4		5		6		7		8	
	B_6PO_4															
	+	−	+	−	+	−	+	−	+	−	+	−	+	−	+	−
Dopa	0	0	8	0	0	0	—	14	—	12	—	14	7	—	20	0
5-HTP	3	5	3	2	0	—	—	20	—	30	—	20	0	—	7	2
e-Dops	11	13	17	6	12	4	—	20	—	4	—	20	25	—	0	0
Pops	0	14	—	—	0	0	—	19	—	2	—	15	—	—	—	—
m-Tyo	21	8	—	—	0	0	41	—	37	—	—	—	0	—	29	0
Mops	0	7	—	—	0	0	—	30	—	—	—	30	—	—	—	—
Hd	7	15	11	11	12	23	—	—	—	12	—	—	0	—	15	0
PS	—	—	—	—	—	—	—	31	—	9	—	31	—	—	—	—

Each Warburg flask contained, in its main compartment, 2 ml. of 1:1 whole homogenate, 40 μg. of B_6PO_4, 1.5 ml. of 0.1 *M* PO_4 buffer at *p*H 7.4, and H_2O to 4.5 ml. Side arm 1 contained 10 μmoles amino acid, and side arm 2 contained 0.2 ml. of 3*N* H_2SO_4. N_2 gas, 1.5 hours, at 37° C.

* Lyophilized supernate of 1:1 homogenate.

† 100 μg. of isobutylisonicotinic hydrazide.

TABLE 2

EFFECT OF THE INHIBITORY SUBSTANCES, 5-HTP AND DOPA DECARBOXYLASES, FROM THE *OCTOPUS APOLLYON* POSTERIOR SALIVARY GLAND ON HOG KIDNEY

	5-HTP		Dopa	
	CO_2 (μl.)	Inhibition (%)	CO_2 (μl.)	Inhibition (%)
Kidney only	99		221	
Kidney + homogenate	70	30	165	25
Kidney + dialyzed homogenate	76	24	—	—
Kidney + lyophyl supernatant	89	11	171	23
Kidney only	118		66	
Kidney + supernatant of boiled extract	116	0	66	0
Kidney only	103		97	
Kidney + ppt of boiled extract	107	0	110	0

Dialysis time 6 hours.

The Warburg vessels contained 1 ml. (30 mg. dry wt.) of lyophilized hog kidney extract; 1 ml. of homogenate (1:2); 1.5 ml. of 0.1 *M* PO_4 buffer at *p*H 7.4; 40 μg. of B_6PO_4. Side arm 1 contained 10 μmoles of 5-HTP or Dopa; side arm 2 contained 0.2 ml. of 3 *N* H_2SO_4. N_2 gas, 1.5 hours, at 37° C.

The effect of lyophilization on the enzyme activity of whole homogenate is shown in TABLE 4.

The decrease in the activity on lyophilization of the most active enzymes in the fresh homogenate indicates that lyophilization cannot be used for storage or purification of the enzymes, with the possible exception of Tyo decarboxylase. It also is of interest that lyophilization lowers the *p*H of the protein solution, necessitating *p*H readjustments.

There is a general indication that B_6PO_4 is a necessary coenzyme for all the decarboxylations studied. TABLE 5 shows manometric activities of the 35 to 45 per cent ammonium sulfate fraction on several substrates.

It can be seen that lyophilization still destroys activity and lowers the *p*H. The effect of added B_6PO_4 on the decarboxylation of Dopa, Dops, and 5-HTP, and the reverse effect on Hd, *m*-Tyo, Pops, and Tyo may be significant in showing differences in enzymes or affinity for the cofactor.

TABLE 6 shows preliminary attempts to isolate the enzymes in separate ammonium sulfate fractions, and to study the effect of *p*H on each.

The *p*H of each enzyme before incubation is shown in the horizontal column at the top of the table. The buffer *p*H values, and thus the *p*H of the enzyme-substrate incubation, appears below this. In general it would seem that an incubation *p*H of 6.5 is most desirable for Dopa, 5-HTP, and *m*-Tyo. Hd decarboxylation seems to be the exception, since two of its highest activities

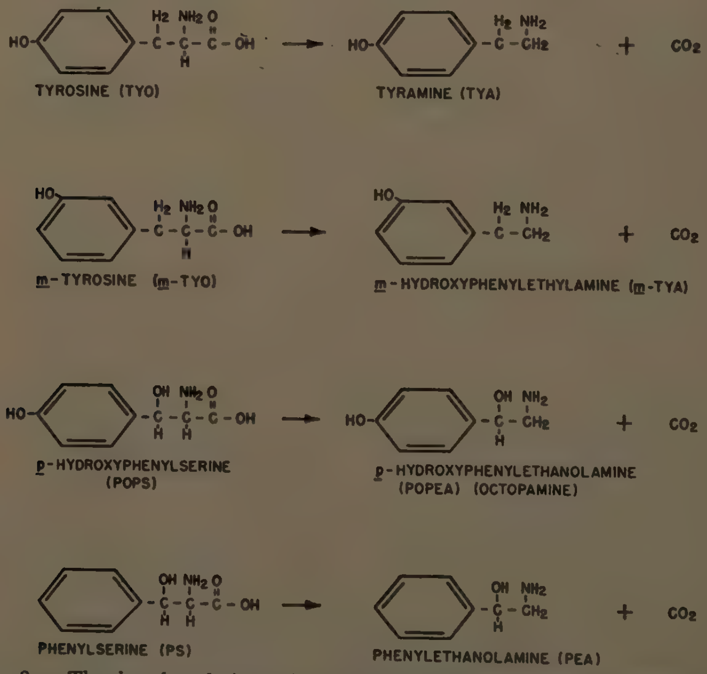
TYROSINE (TYO)
TYRAMINE (TYA)
+ CO2
m-TYROSINE (m-TYO)
m-HYDROXYPHENYLETHYLAMINE (m-TYA)
+ CO2
p-HYDROXYPHENYLSERINE (POPS)
p-HYDROXYPHENYLETHANOLAMINE (POPEA) (OCTOPAMINE)
+ CO2
PHENYLSERINE (PS)
PHENYLETHANOLAMINE (PEA)
+ CO2

FIGURE 8*a*. The decarboxylations of the posterior salivary glands. The substrates were L-tyrosine; DL-*m*-tyrosine; DL-*erythro*-*p*-hydroxyphenylserine; DL-*erythro*-phenylserine; L-histidine; L-tryptophan; DL-5-hydroxytryptophan; L-3,4-dihydroxyphenylalanine; DL-*erythro*-3,4-dihydroxyphenylserine; and DL-*erythro*-*m*-hydroxyphenylserine. (*Above* and *opposite*)

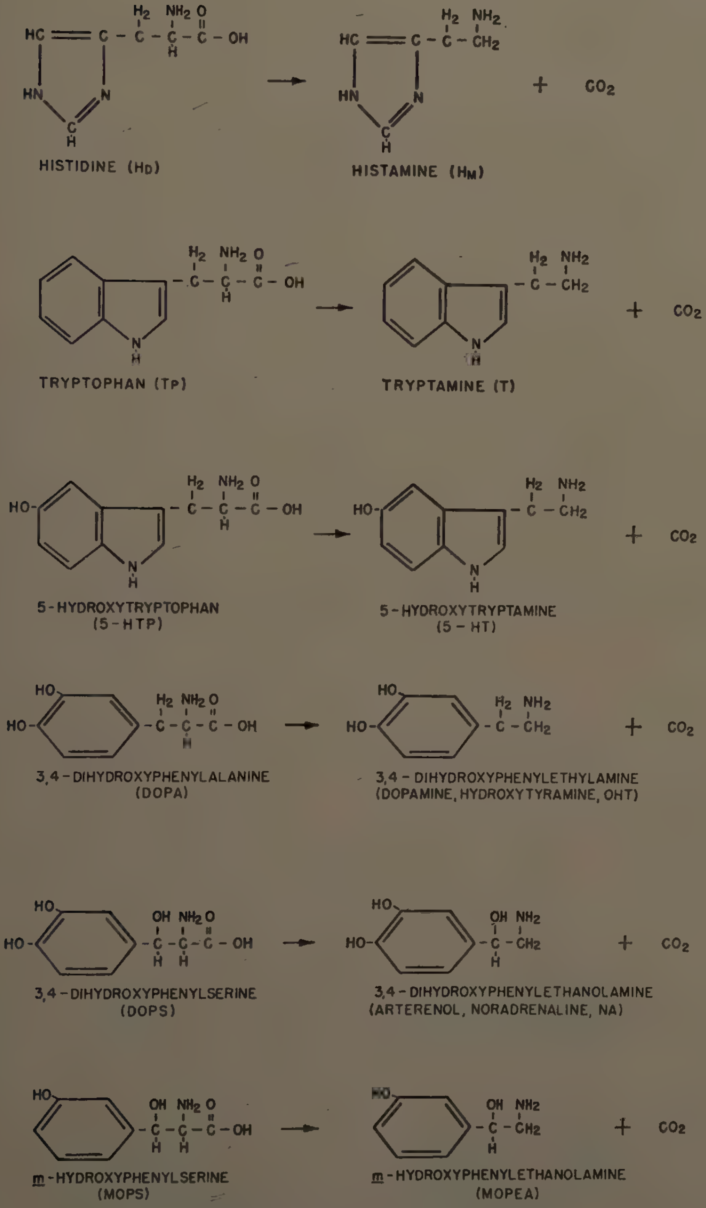
HISTIDINE (HD)
HISTAMINE (HM)
+ CO2
TRYPTOPHAN (TP)
TRYPTAMINE (T)
5-HYDROXYTRYPTOPHAN (5-HTP)
5-HYDROXYTRYPTAMINE (5-HT)
3,4-DIHYDROXYPHENYLALANINE (DOPA)
3,4-DIHYDROXYPHENYLETHYLAMINE (DOPAMINE, HYDROXYTYRAMINE, OHT)
3,4-DIHYDROXYPHENYLSERINE (DOPS)
3,4-DIHYDROXYPHENYLETHANOLAMINE (ARTERENOL, NORADRENALINE, NA)
m-HYDROXYPHENYLSERINE (MOPS)
m-HYDROXYPHENYLETHANOLAMINE (MOPEA)

FIGURE 8*b*. See legend on opposite page.

are at *p*H 7.0. Several Warburg flask contents, shown in TABLE 6, were saved and run through a cotton-acid-succinate column, and the wash and eluate (elutrient 0.1 to 3.0 *N* HCl, depending on the amine sought) were reduced in volume, spotted on Whatman No. 4 paper, and developed by descending technique with isopropanol-acetic acid-water (70:5:25 v/v/v). The chromatograms were sprayed with sulfanilic acid and *N*,2,6-trichloro-*p*-benzoquinoneimine-borate buffer. As shown in TABLE 7, traces of end products such as 5-HT and *m*-Tya were consistently found; OHT and Hm were unequivocal. The 5-HT and Hm formed by action of ammonium sulfate fractions II and IV, however, are quite significant, since all endogenous amines were previously removed by dialysis.

TABLE 3

EFFECT OF AMMONIUM SULFATE ON 5-HYDROXYTRYPHAN DECARBOXYLASE FROM *OCTOPUS APOLLYON* POSTERIOR SALIVARY GLAND

Dialysis time	*p*H	CO_2 evolved (µl.)*
3	6.2	4
6	6.2	4
7	6.4	11
8	6.5	12
8.5	6.5	62
8.5	7.9	64

The Warburg vessel contained, in its main compartment, 2 ml. of dialysate fraction II 30–47% ppt; 1.5 ml. of 0.1 *M* PO_4 buffer, *p*H 6.5; 20 µg. of B_6PO_4; and H_2O to 4.5 ml. Side arm 1 contained 10 µm. of 5-HTP; side arm 2 contained 0.2 ml. of 3 *N* H_2SO_4. N_2 gas, 1.5 hours, at 37° C.

* Triplicate, control enzyme plus substrate killed with 0.2 ml. of 0.001 *M* $CuSO_4$.

TABLE 4

EFFECT OF LYOPHYLIZATION ON AMINO ACID DECARBOXYLASE ACTIVITY OF *OCTOPUS APOLLYON* POSTERIOR SALIVARY GLAND

	Fresh (*p*H 6.5)		Lyophilized			
			Enzyme unadjusted (*p*H 5.6)		Enzyme adjusted (*p*H 6.4)	
B_6PO_4	+	−	+	−	+	−
Dopa	1	26				
e-Dops	81	87	4	4	31	25
5-HTP	84	11	0	—	26	11
Hd	3	3				
m-Tyo	10	1				
Pops	12	—				
Tyo	1	11	12	0	21	4

Each vessel contained, in its main compartment, 100 mg. dry weight of whole homogenate of posterior salivary gland enzyme or its equivalent wet weight; 2.5 ml. of 0.1 *M* PO_4 buffer, *p*H 6.5; 40 µg. of B_6PO_4 as indicated; and H_2O to 4.5 ml. Side arm 1 contained 10 µmoles of amino acid; side arm 2 contained 0.2 ml. of 3 *N* H_2SO_4. N_2 gas, 1.5 hours, at 37° C.

TABLE 8 shows a peculiar type of subfractionation caused by precipitation of various substances, possibly protein, with continued dialysis of the 52 to 72 per cent ammonium sulfate fractions. When the dialysate mixture was removed from the dialysis bag after eight hours dialysis, it was centrifuged to determine if the activity was in the supernatant or the precipitate. It was then noticed that the precipitate had formed into three layers that were colored red, white, and blue. In an attempt to redissolve the separate layers in 0.01

TABLE 5

EFFECT OF LYOPHYLIZATION ON DECARBOXYLASE IN AMMONIUM SULFATE FRACTION OF *OCTOPUS APOLLYON* POSTERIOR SALIVARY GLAND

	Fresh (FR 35–45%) (*p*H 6.7)		Lyophilized (FR 35–45%)			
			Enzyme unadjusted (*p*H 5.6)		Enzyme adjusted (*p*H 6.4)	
B_6PO_4	+	−	+	−	+	−
Dopa	34	0				
e-Dops	105	9	9	22	0	0
5-HTP	87	22	0	0	23	1
Hd	25	68				
m-Tyo	25	124				
Pops	37	62				
Tyo	93	217	0	0	16	0

Each vessel contained, in its main compartment, 100 mg. dry weight or equivalent wet weight of enzyme; 2.5 ml. of 0.1 *M* PO_4 buffer, *p*H 6.5; 40 µg. of B_6PO_4 as indicated; and H_2O to 4.5 ml. Side arm 1 contained 10 µmoles of amino acid; side arm 2 contained 0.2 ml. of 3 *N* H_2SO_4. N_2 gas, 1.5 hours, at 37° C.

TABLE 6

EFFECT OF *p*H ON DECARBOXYLATION OF SUBSTRATES

Substrate	Supernate (H₂O extract of gland)				Enzyme fractions									
	Enzyme (*p*H 6.4)				I (0–30%) (*p*H 5.5)		II (30–42%) (*p*H 5.7)		III (42–47%) (*p*H 6.4)		IV (47–52%) (*p*H 5.8)		V (52–57%) (*p*H 5.5)	
	Buffer *p*H													
	6.0	6.5	7.0	7.5	6.5	7.0	6.5	7.0	6.5	7.0	6.5	7.0	6.5	7.0
Dopa	0	39	26	13	0	15	17	8	9	4	3	10	31	9
5-HTP	5	25	18	2	0	3	34	7	23	21	2	12	9	14
m-Tyo	18	55	19	21	27	20	32	14	46	4	12	11	27	12
Hd	16	27	0	0	1	2	30	100	47	23	0	86	38	22
e-Dops											9			
t-Dops											22*			

The Warburg vessel contained, in its main compartment, 2 ml. of supernate of 1:2 homogenate or dialysate; 1.5 ml. of 0.1 PO_4 buffer, *p*H as indicated; 40 µg. B_6PO_4; and H_2O to 4.5 ml. Side arm 1 contained 10 µmoles of substrate; side arm 2 contained 0.2 ml. 3 *N* H_2SO_4. N_2 gas, 1.5 hours, at 37° C.

* Fractionated on alumina and Dowex CG-50 columns. Optical density graphed and paper chromatographed.

M phosphate buffer, *p*H 6.5, it appeared that the red layer was readily soluble and could thus be separated from the blue and white layers, which were soluble with difficulty and were suspended together. It can be seen that the supernatant of whole homogenate has weak activity and that the fraction is quite active. The supernatant of the dialyzed fraction seems very active, but the red layer, being only a few milligrams of estimated weight, probably is much more active than indicated. A careful study of the activity of this layer, expressing the results on the basis of Kjehldahl nitrogen determinations, is in progress. The blue and white layers also may be more active than the table indicates, due to their small quantities.

Table 7

Fraction	Substrate	*p*H	CO_2 evolved (µl.)	Chromatography
Homogenate	*m*-Tyo	6.5	55	TR, *m*-Tya
Homogenate	Dopa	6.5	39	OHT
Homogenate	5-HTP	6.5	25	TR, 5-HT
FR II (30–42%)	5-HTP	6.5	34	TR, 5-HT
FR IV (47–52%)	Hd	7.0	86	Hm

The manometric method was the same as that in TABLE 6.

The column chromatography employed a 100-mg. CAS column, 20 ml. H_2O wash, and a 20 ml. 1 *N* HCl eluate. TR indicates trace.

The paper chromatography employed Whatman No. 4 paper; a solvent consisting of isopropanol-acetic acid-water (70:5:25 v/v/v); and sprays of sulfanilic acid and *N*,2,6-trichloro-*p*-benzoquinoneimine-borate buffer, in a 2 per cent solution, *p*H 9.5.

Table 8

Subfraction of IV by Centrifugation of Dialysate

Substrate	H_2O homogenate (1:2 supernatant) (µl. CO_2)	Fraction IV (52–57%)							
		Buffer dialysis (µl. CO_2)				H_2O dialysis (µl. CO_2)			
		1	2	3	4	1	2	3	4
Dopa	7	24	22	6	35	10	19	17	6
t-Dops	25	30	32	4	6	23	1	11	15
5-HTP	0	22	11	17	0	39	1	0	3
Hd	0	6	8	23	5	16	17	11	5
m-Tyo	0	16	20	1	—	8	10	10	—
*p*H Enzyme	6.5	6.5	7.5	7.0	6.5	6.6	7.1	7.0	6.5

Key: 1, supernate of dialysate; 2, red ppt in 40 ml. of 0.01 *M* PO_4 buffer, *p*H 6.5; 3, blue and white ppt in 40 ml. of 0.01 *M* PO_4 buffer, *p*H 6.5; and 4, whole fraction.

Each vessel contained, in its main compartment, 2 ml. of enzyme (1, 2, 3, 4) as indicated; 1.5 ml. of 0.1 *M* PO_4 buffer, *p*H 6.5; 40 µg. of B_6PO_4; and H_2O to 4.5 ml. Side arm 1 contained 10 µmoles of substrate; side arm 2 contained 0.2 ml. of 3 *N* H_2SO_4. N_2 gas, 1.5 hours, at 37° C.

Since it was noted in TABLE 6 that Hd was most rapidly decarboxylated by the 40 and 50 per cent ammonium sulfate fractions, a radioactive experiment was set up, as shown in TABLES 9 and 10. The methods have been described above.

TABLE 9

DECARBOXYLATION OF C^{14}-HISTIDINE BY TISSUES OF *OCTOPUS BIMACULATUS*

Expt. No.	Organ	*p*H	Cpm minus blank
1	Posterior salivary gland homogenate	6.0	10
2	Posterior salivary gland homogenate	8.0	26
3	None	7.0	0
4	Posterior salivary gland homogenate	7.0	23
5	Posterior salivary gland homogenate	7.5	36
6	Posterior salivary gland slices	7.2 sterile sea water	19
7	Liver	6.5	0
8	Liver	7.0	0
9	Mantle	6.5	5
10	Mantle	7.0	15

Each Dubnoff flask contained 2 ml. of 0.1 *M* PO_4 buffer and 0.2 per cent glucose, *p*H as indicated; 1 ml. of 1:1 tissue homogenate; 20 μg. of C^{14}-ring-2-histidine (200,000 cpm); 100 μg. of aminoguanidine; 40 μg. of B_6PO_4; and 1 drop of toluene. Incubation time 3 hours at 37° C.

TABLE 10

DECARBOXYLATION OF C^{14}-HISTIDINE BY *OCTOPUS APOLLYON* POSTERIOR SALIVARY GLANDS

Expt. No.	Organ	*p*H	Cpm minus blank
1	FR 30–60%	7.0	35
2	FR 30–60%	7.0	27
3	Homogenate	6.0	5
4	Homogenate	6.6	2
5	Homogenate	7.0	1

Each Dubnoff flask contained 2 ml. of 0.1 *M* PO_4 buffer and 0.2 per cent glucose, *p*H as indicated; 2 ml. of 1:1 homogenate or 2 ml. of 30 to 60 per cent ammonium sulfate fraction (43 mg.); 20 μg. of C^{14}-ring-2-histidine (200,000 cpm); 100 μg. of aminoguanidine; and 40 μg. of B_6PO_4. Incubation time 3 hours at 37° C.

TABLE 11

MANOMETRIC ESTIMATION OF AMINO ACID DECARBOXYLASES IN POSTERIOR SALIVARY GLAND OF *OCTOPUS BIMACULATUS*

Expt. No.	1		2		3		4*	
	B_6PO_4							
	+	−	+	−	+	−	+	−
Dopa	55	18	81	—	235	18	33	0
5-HTP	—	—	0	—	—	—	0	0
Hd	—	—	8	—	—	—	—	—
Tyo	0	0	—	—	—	—	—	—
Pops	0	0	—	—	—	—	—	—
e-Dops	—	—	—	—			5	0
m-Tyo	—	—	—	—			26	0

Each vessel contained in its main compartment 2.0 ml. of supernate 1:1 homogenate; 1.5 ml. of 0.1 *M* PO_4 buffer, *p*H 7.0; 40 μg. of B_6PO_4 phosphate; and H_2O to 4.5 ml. Side arm 1 contained 10 μmoles of substrate; side arm 2 contained 0.2 ml. of 3 *N* $HClO_4$. N_2 gas, 1 hour, at 30° C.

* L-Tryptophan in place of 5-HTP.

The 30 to 60 per cent fraction shows definite activity slightly higher, at a *p*H of 7.0 (TABLE 10). The homogenates are inactive at all *p*H values tried. The homogenates of the smaller *O. bimaculatus*, however, show some activity, as illustrated in TABLE 9, with optimum *p*H possibly at 7.5.

The use of sterile sea water at *p*H 7.2 instead of phosphate buffer made little difference in the activity of the enzyme. Liver and mantle homogenates at *p*H 6.5 and 7.0 had very low or insignificant activity. The activity of whole homogenate of the posterior glands of *O. bimaculatus* and the absence of activity of those of *O. apollyon* may be due either to the freshness of the former (used immediately after death, unfrozen), or to species differences.

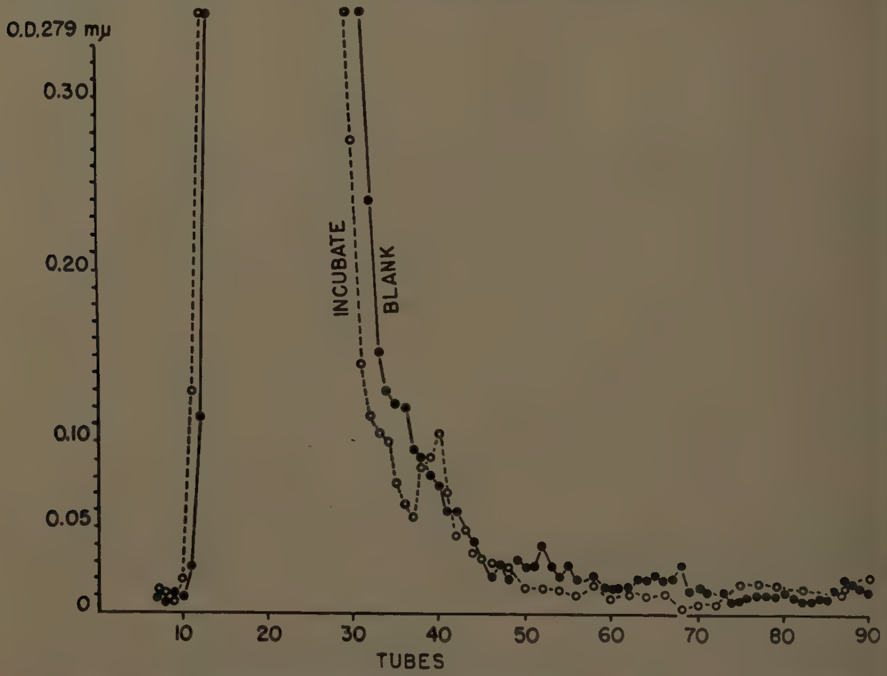

FIGURE 9. The Warburg flask reaction in Experiment 3, TABLE 11, of *O. bimaculatus* posterior salivary glands and their Dopa-decarboxylase activity. The killed enzyme-substrate blank and the incubated enzyme and substrate are plotted with optical density at 279 mμ on the ordinates, and the numbers of tubes on the abscissas.

In a survey of decarboxylase activity of the *O. bimaculatus* glands, shown in TABLE 11, it should be noted that activity is found only with Dopa, Dops, and *m*-Tyo, which are all presumably decarboxylated by the same enzyme. The other substrates, however, show low activities by radioactive methods. The high activity of Dopa decarboxylase again may be due to the freshness of the tissue of *O. bimaculatus*, or indeed to true species differences, since freezing in general does not appreciably diminish the activity of Dopa decarboxylase in most other tissues.

To identify further the reaction in the Warburg flask in Experiment 3 of TABLE 11, the proteins were precipitated with $HClO_4$ and filtered off. Excess

$HClO_4$ was precipitated as $KClO_4$; the supernatant was adjusted to *p*H 6.1 with 0.2 *N* ammonium acetate, put on a 1 × 35 cm. Dowex CG-50 column, and eluted with 0.4 *N* ammonium acetate, *p*H 6.1, at a rate of 4 ml./hour, 1 ml. per tube, on the automatic fraction collector. FIGURES 9 and 10 show the killed enzyme-substrate blank and the incubated enzyme and substrate plotted with optical density at 279 mμ on the ordinates, and the numbers of tubes on the abscissas.

The O.D. peak after 112 ml. elution volume was superimposable on a peak representing a small endogenous dopamine content, and corresponded to 8 mmole of dopamine, which agreed closely with the CO_2 production of 235 μl. in the Warburg, indicating a nearly complete reaction.

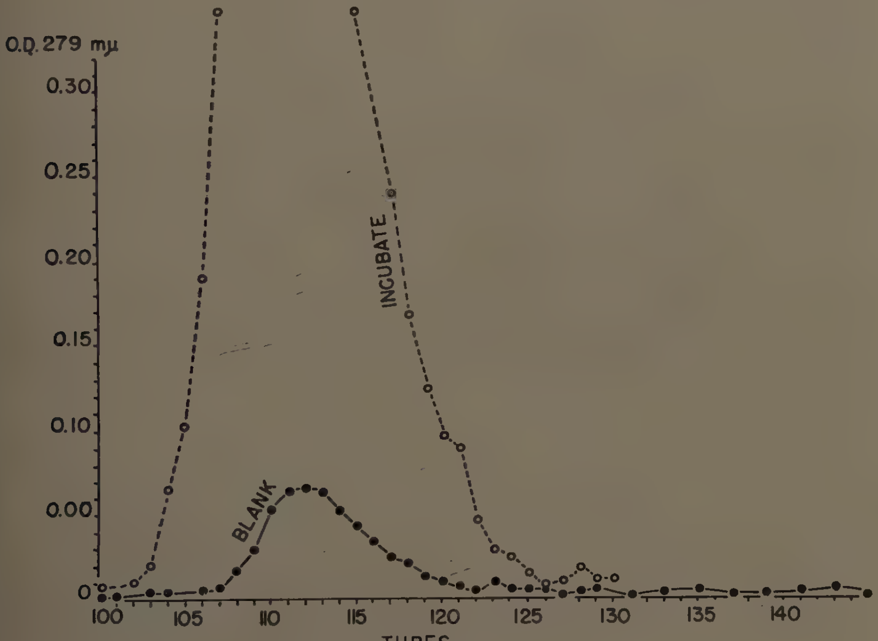

FIGURE 10. See legend for FIGURE 9.

A further investigation of the Dopa decarboxylase activity of the posterior salivary glands of *O. bimaculatus* was made, incubating 1 ml. of supernatant of a 1:2 homogenate with 1.5 × 10^6 cpm of C^{14}-Dopa and 1 mg. of unlabeled Dopa for three hours at *p*H 7.0. The mixture also contained 40 μg. of isobutylisonicotinic hydrazide. FIGURE 11 shows the results graphed as O.D. and cpm. The radioactive peak falls exactly over the peak of 200 μg. of unlabeled dopamine added to the column for peak identification. The pooled fractions from tubes 51 to 75 were evaporated to a small volume at reduced temperature and pressure and spotted on Whatman No. 1 paper and developed with isopropanol-acetic acid-water (70:5:25 v/v/v). Strips, 1 × 2 cm., were cut from the dried chromatogram and counted in the automatic flow counter. As shown in FIGURE 12, a radioactive peak was found directly over the spot of

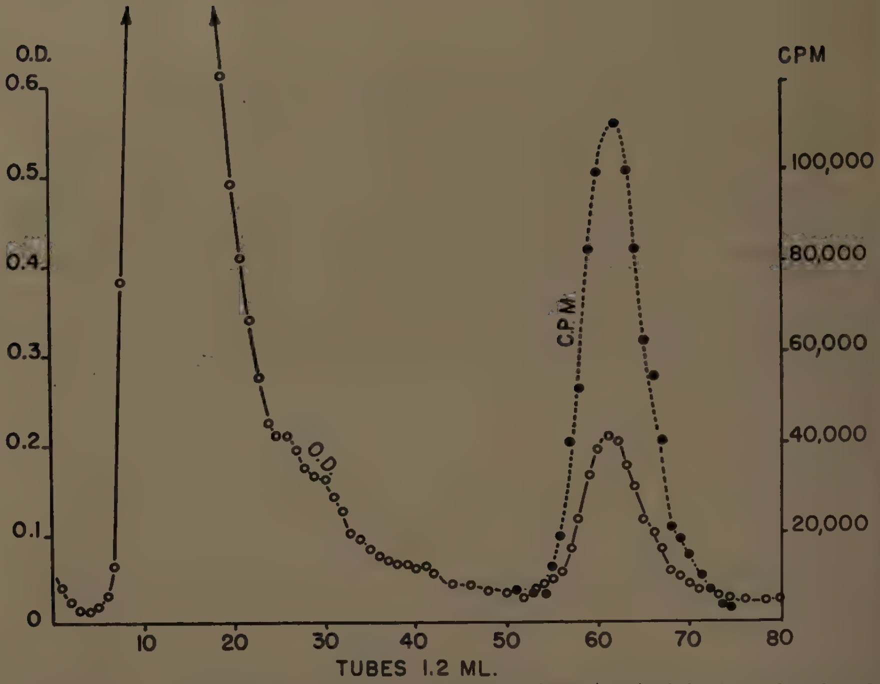

FIGURE 11. Optical density (O.D.) and counts per minute (cpm) of the Dopa-decarboxylase activity of the posterior salivary glands of *O. bimaculatus*, determined by incubating 1 ml. of supernatant of a 1:2 homogenate with 1.5 × 10^6 cpm of C^{14}-Dopa and 1 mg. of unlabeled Dopa. The radioactive peak falls exactly over the peak of 200 μg. of unlabeled dopamine added to the column for peak identification.

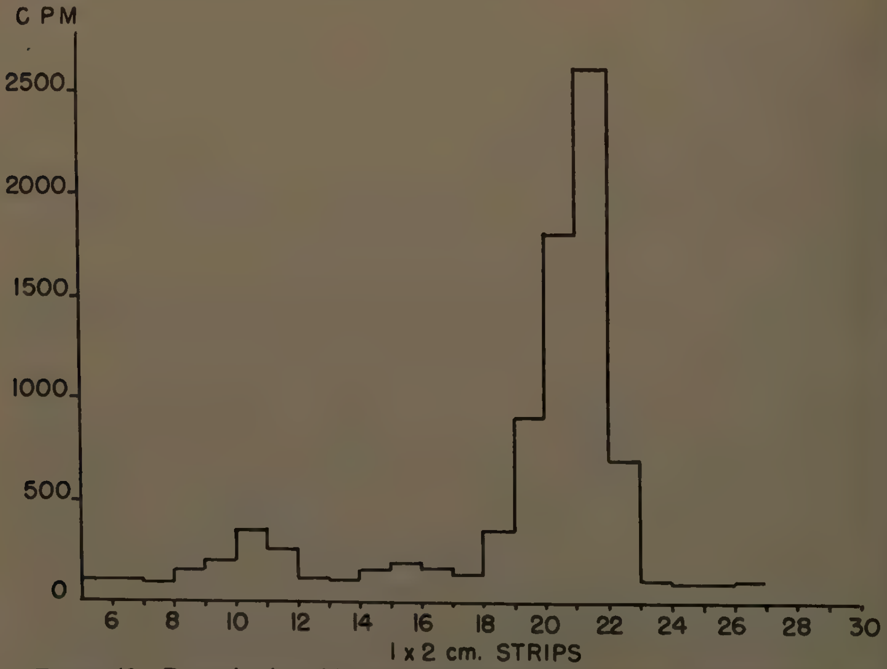

FIGURE 12. Determination of Dopa-decarboxylase activity of 1 × 2 cm. strips cut from the dried chromatogram and counted in the automatic flow counter. A radioactive peak was found directly over the spot of dopamine, as shown by spraying with the potassium ferricyanide-ferric sulfate reagent.

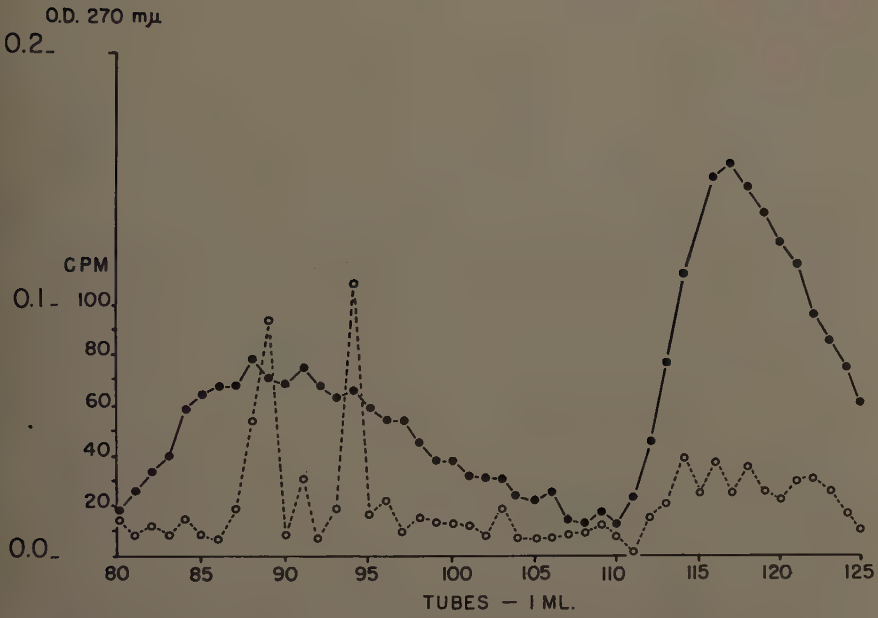

FIGURE 13. Determination of C^{14}-tyrosine activity in *O. bimaculatus* glands, similar to the experiment illustrated in FIGURE 12. A 1 × 35 cm. Dowex CG-50 column, *p*H 7.0, was used with 1 gm. wet weight of the glands. Three O.D. peaks and four radioactive peaks resulted.

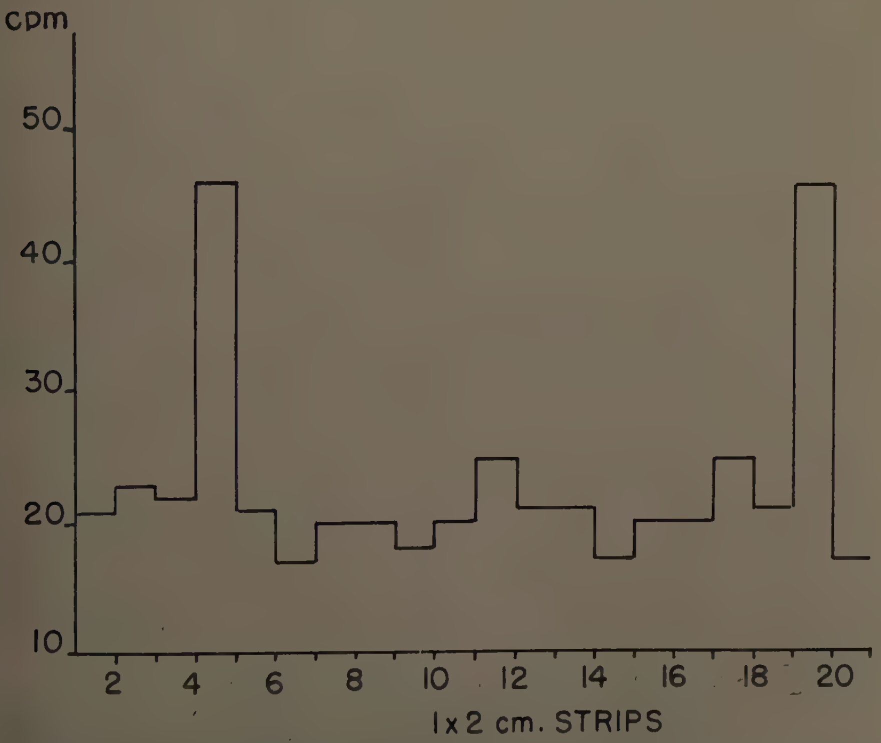

FIGURE 14. Optical density peak No. 1, in FIGURE 13, showed radioactivity in the Tyo spot when paper chromatographed, while peak No. 2, spotted from the pooled lot of tubes 80 to 105, gave two radioactive peaks.

dopamine, shown by spraying with the potassium ferricyanide-ferric sulfate reagent.

A similar experiment with *O. bimaculatus* glands using C^{14}-Tyo as substrate gave three O.D. peaks and four radioactive peaks. Optical density peak No. 1 (tubes 12 to 50), when paper chromatographed, showed radioactivity in the Tyo-spot, while peak No. 2, spotted from a pooled lot of tubes 80 to 105 (FIGURE 13) gave two radioactive peaks, as shown in FIGURE 14. This figure was obtained by counting 1 × 2 cm. strips cut out of a paper chromatogram spotted

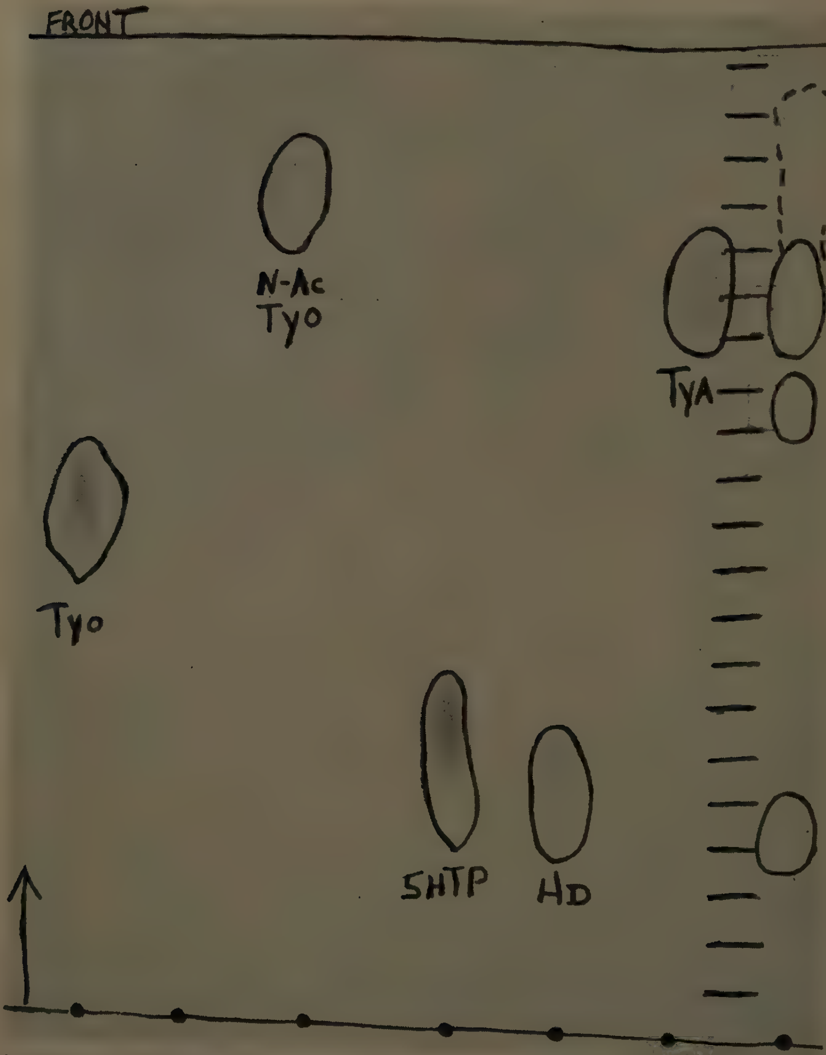

FIGURE 15. The results illustrated in FIGURE 14 were determined by counting 1 × 2 cm. strips from this paper chromatogram, spotted with a concentrate of the fractions from peak No. 2 (tubes 80 to 105). The *O. bimaculatus* posterior salivary glands were incubated with C^{14}-tyrosine.

with a concentrate of the fractions from peak No. 2 (tubes 80 to 105). This chromatogram is shown in FIGURE 15.

Strip No. 5 was slightly radioactive and fell in the amino acid range on the chromatogram, but was not Tyo, a phenomenon that might be explained by the formation of Dopa. Strip No. 19 also was radioactive, and fell on the chromatogram just ahead of the Tya R_f. It could be a conjugated Tya or possibly a Tya metabolite such as *p*-hydroxyphenylacetic acid. The peak is unknown and is under investigation. According to previous experience with the characteristics of this column, peak No. 3 (tubes 110 to 125) falls in the fractions usually occupied by NA.

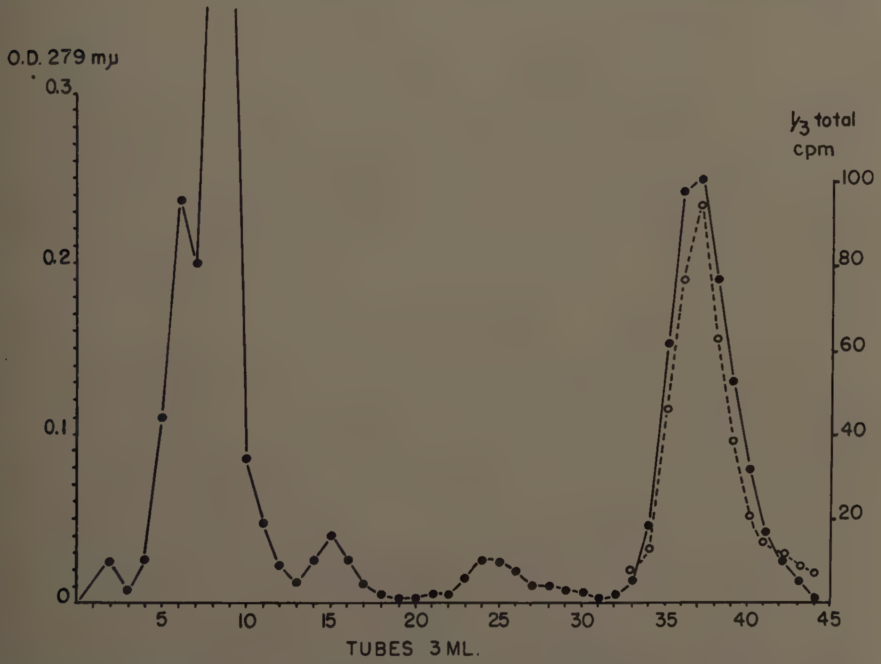

FIGURE 16. Results of fractionation of the catechols found after incubating a 1:1 homogenate of *O. apollyon* posterior salivary glands for three hours at 37° C. with 400,000 cpm of C^{14}-Dops in the presence of 40 μg. of B_6PO_4, 100 μg. of isobutylisonicotinic hydrazide, and 1 mg. of unlabeled Dops in 0.1 *M* phosphate buffer at *p*H 7.2, with 2 drops of toluene as a bactericide.

Although the tissues of *O. apollyon* had been frozen, shipped, and thawed, an experiment with C^{14}-Dops as substrate showed definite decarboxylase activity, in confirmation of the preceding Warburg data. FIGURE 16 shows the results of fractionating the catechols found after incubating a 1:1 homogenate of *O. apollyon* posterior salivary glands for three hours at 37° C. with 400,000 cpm of C^{14}-Dops in the presence of 40 μg. B_6PO_4, 100 μg. isobutylisonicotinic hydrazide, and 1 mg. unlabeled Dops in 0.1 *M* phosphate buffer at *p*H 7.2, with 2 drops of toluene as a bactericide. It is interesting to note that all flasks that contained toluene showed about three times more activity than duplicates without toluene, even though the latter were contaminated with bacteria that might contain decarboxylases. From the results obtained at *p*H 6.0, 6.6, and 7.2, it would appear that *p*H 6.6 is the optimum for this tissue and decarboxylation, confirming the Warburg results. The radioactivity, as shown in FIGURE 16, falls directly on the peak obtained by adding authentic NA for purposes of identification. The presence of radioactive NA also was confirmed by paper chromatography.

A few amine and decarboxylase studies also have been performed on the anterior salivary glands of *O. apollyon*, but were only preliminary and will be reported elsewhere.

Julius Axelrod of the National Institutes of Health, Public Health Service, Bethesda, Md., generously analyzed frozen posterior salivary gland material from *O. apollyon* for catechol-O-methyl transferase[101] and imidazole *N*-methyl transferase.[102] The soluble supernatant fraction of a homogenate O-methylated 33 mμ *M* per gram of Epi to metanephrine in one hour. From these results it would appear that if the gland makes more than 50 μg. Epi, dopamine, or NA per hour per gram *in vivo*, this enzyme activity is weak, but if it makes 10 μg. or less, it could metabolize all the catechols that are made. There was no imidazole *N*-methyl transferase activity.

Summary

(1) The posterior salivary glands of *Octopus apollyon* and *Octopus bimaculatus* have been examined for their amine and amino acid decarboxylase content. A preliminary survey of the latter in *O. apollyon* by Warburg manometric methods showed decarboxylation of L-3,4-dihydroxyphenylalanine (Dopa), DL-5-hydroxytryptophan, DL-*erythro*-3,4-dihydroxyphenylserine, DL-*erythro*-*p*-hydroxyphenylserine, DL-*m*-tyrosine, DL-*erythro*-*m*-hydroxyphenylserine, L-histidine, and DL-*erythro*-phenylserine.

(2) Studies were made of the effects on decarboxylase activity of ammonium sulfate fractionation, pyridoxal-5-phosphate, *p*H, and dialysis.

(3) The decarboxylation of 3,4-dihydroxyphenylserine was also studied, using C^{14}-labeling and radiochemical techniques. *O. apollyon* glands rapidly decarboxylate this substrate.

(4) Histidine and *m*-tyrosine also are decarboxylated by *O. bimaculatus* glands, as shown by the Warburg manometric method.

(5) Dopa decarboxylase activity was demonstrated by photometric analysis after column chromatography of incubates of *O. bimaculatus* gland homogenates, and verified by radioisotope analysis of column fractions and by paper chromatographic methods.

(6) Tyrosine decarboxylation was demonstrated in the glands of *O. bimaculatus* by the isotope technique.

(7) Histidine decarboxylation was demonstrated in the glands of both species by the isotope method.

(8) Endogenous amines found in the posterior salivary glands of both species include octopamine, serotonin, "serotonin decomposition product", histamine, and dopamine. The latter has not been previously described as a constituent.

(9) Tyrosine and histidine were found as endogenously occurring amino acids in the posterior salivary glands of both species.

(10) Many unidentified R_fs were found by paper chromatography. Preliminary evidence indicates that among them may be adrenaline, noradrenaline, and dopacetic acid.

Acknowledgments

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SOME CHEMICAL PROPERTIES OF NEREISTOXIN*

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Among anglers it has been a well-known fact that some carnivorous insects, such as the fly and ant, die from contact with the dead body of a marine annelid, *Lumbriconereis heteropoda* Marenz, which is commonly used as a bait. In 1934 Nitta[1] isolated a neurotoxic principle from this worm and named it nereistoxin. It was obtained in faintly yellow crystals of hydrogen oxalate (m.p. 178∼180° C.) of unpleasant odor, soluble in fat solvents and insoluble in water. From analyses of hydrogen oxalate and picrate (m.p. 170° C.), the empirical formula was postulated to be $C_5H_9NS_2$. Little knowledge was obtained on the functional groups, whereas it was found that the toxin responds to most alkaloid reagents, reduces the Fehling and ammoniacal silver nitrate solutions, and loses toxicity when treated with potassium permanganate. Nitta, however, turned his attentions to the pharmacological aspects, giving no further consideration to the chemical ones, which have been left untouched by scientists thus far.

By using about 10 species of test animals from monkey to fly, Nitta[2] concluded that the toxin exerts a strong action, chiefly on the nervous system, and slightly affects the internal organs and blood. In injected animals, myosis, enhanced movement of the intestinal smooth muscle, shedding tears, and slavering were observed as typical symptoms. The small dose accelerated heart action, whereas the large dose repressed it; the repression effect was somewhat diminished by atropine. The lethal dose was 0.38 mg./10 gm. for mice and 1.8 mg./kg. for rabbits in subcutaneous injection. Recently, Matsue and Hirayama† reported that fish are more susceptible than warm-blooded animals, and the toxicity for fish is considerably enhanced by adjusting the medium to the alkaline side.

In light of the increasing importance of organic sulfur compounds in biological materials[3] and the striking peculiarity of the empirical formula of nereistoxin, we recently commenced this study. Results thus far obtained in elemental analyses, qualitative tests on functional groups, estimation of ultraviolet and infrared absorption spectra, and reduction with sodium borohydride or Raney nickel have demonstrated that the toxin is a tertiary amine possessing a cyclic disulfide ring. These results have led us to postulate the possible structural formula, as discussed later, which has a 1,2-dithiolane moiety similar to 6:8-thioctic acid (α-lipoic acid or 1,2-dithiolane-3-valeric acid), a member of the vitamin B group.

Extraction of Nereistoxin

The extraction method of Nitta[1] was modified. The worms, collected in the spring tide from the bed of Tokyo Bay, were about 40 cm. in length and 0.5 cm. in diameter. Live materials (1 kg.) were killed by boiling with 2.5 per cent acetic acid (1 l.) for 15 min. and minced in a mortar after centrifugation. The residue was twice extracted with boiling water (3 l. total). The combined extracts were condensed *in vacuo*, to which methanol (3 l.) was added to remove impurities as white precipitates. The supernatant was again condensed to 500 ml., defatted with ether by shaking or in a liquid-liquid extraction apparatus, and adjusted to *p*H 9.0 with sodium hydroxide solution to extract the toxin by shaking with each 150 ml. portion of chloroform three times. The toxin was then transferred from chloroform into dilute hydrochloric acid and again returned to ether in the same manner. After desiccation with anhydrous sodium sulfate, most of the ether was driven off. On addition of a half-saturated ether solution of oxalic acid, nereistoxin hydrogen oxalate (m.p. 167° C.) precipitated in faintly yellow, thin flakes. The average yield was about 73 mg. from 1 kg. of fresh material and was almost quantitative in toxicity when assayed with a killifish, *Oryzias latipes*.

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* *Studies on Marine Biotoxins.* I.

† Unpublished data.

Analysis of Nereistoxin

Nereistoxin hydrogen oxalate. Crude oxalate (224 mg.) was recrystallized twice from 95 per cent alcohol, yielding 139 mg. with a m.p. (decomp.) of 168∼170° C. Components, in percentages, were determined by analysis to be C, 35.15; H, 5.16; N, 5.78; and S, 26.38; as calculated theoretically for $C_7H_{13}NO_4S_2$ they were C, 35.13; H, 5.46; N, 5.85; and S, 26.79. By titration with N/20 NaOH the molecular weight was determined to be 244, compared with a calculated weight for $C_7H_{13}NO_4S_2$ of 239. The material was soluble in water, less soluble in alcohol, and insoluble in most fat solvents. In paper chromatography the hydrogen oxalate was confirmed to be pure, and gave only one spot. The R_f value was 0.60 (BuOH:AcOH:H_2O, 4:1:5), 0.08 (BuOH saturated with H_2O), 0.91 (EtOH:concentrated NH_4OH, 95:5), and 0.82 (BuOH: pyridine:H_2O, 4:3:7) in ascending. The toxicity for *Oryzias latipes* varied according to the *p*H of the medium, and was stronger at the alkaline side. The minimum lethal concentration was about 0.6 ppm at *p*H 7.6∼8.0.

Free base. A colorless liquid of no objectionable odor, with a b.p. of 212–213° C., was distilled from alkaline solution with steam or by aeration at 80° C. It was soluble in most organic solvents, and behaved similarly to tertiary polyamines of five carbons in celite partition chromatography,[4] which may serve as a purification method. Polymerization to a gummy substance was once experienced in evaporation of the ether solution in a stream of nitrogen.

Picrate. The material was recrystallized twice from 97 per cent alcohol, yielding fine needles with a m.p. of 169° C. Analysis revealed 14.26 per cent N, compared with 14.81 per cent calculated for $C_{11}H_{14}N_4O_7S_2$.

Color Reactions

Functional groups were examined in spot tests with reagents prepared in the manner described by Feigl[5] or Block.[6]

The nitrogen in nereistoxin was found to be present in the form of tertiary or quaternary amine by positive reaction with the Dragendorff and Mecke reagents, and by negative reaction with the ninhydrin, Rimini, Sakaguchi, and diazo reagents.

With the sulfur group some ambiguous results were obtained in an earlier experiment, in which a too-diluted test solution was used. Later tests were carried out more strictly, and the following sulfur compounds were used as reference: thiamine hydrochloride, synthesized 5-methyl-2-mercapto-2-thiazoline,[7] cysteine, cystine, methionine, sodium dimethyldithiocarbamate, thiourea, allylisothiocyanate, and thioctic acid. Nereistoxin was positive for platinic iodide reagent, and negative for the Grote and sodium nitroprusside reagents for thiol, thioether, and thioketone. In Feigl's sodium azide:iodine reaction, slightly delayed evolution of nitrogen gas was observed. In the sodium nitroprusside test for disulfide, positive pink color developed after heating at 70° C., similar to the results with thioctic acid, but differing slightly from the reaction with cystine, which showed instant coloration without heating. These results indicate that two atoms of sulfur form the disulfide, probably a cyclic one.

In addition nereistoxin gave a black spot after extended heating with ammoniacal silver nitrate, and the test for the allyl group was negative.

Ultraviolet and Infrared Absorption Spectra

In the ultraviolet region the free base showed a weak but very significant absorption peak at 330 mμ (ϵ 207) and the hydrogen oxalate at 320 mμ (ϵ 144), as shown in FIGURE 1. The absorption pattern may offer an important basis for the postulation of structure, which will be discussed later.

Infrared spectra were examined on the hydrogen oxalate and free base; FIGURE 2 shows the absorption pattern of the latter, which is more adequate for the characterization of functional groups. Bands at 2980, 2940, and 2870 cm.$^{-1}$ may indicate the presence of CH_3—, —CH_2—, and —CH— groups,[9] and two bands at 2830 and 2780 cm.$^{-1}$ may indicate the group —$N(CH_3)_2$.[10] The absorption peaks near 1440 cm.$^{-1}$ may be attributable to the above groups, and those at 1048 or 1003 cm.$^{-1}$ to the stretching vibration of —C—N-linkage. Many peaks in the 1360~900 cm.$^{-1}$ region are difficult to interpret. Absorption bands arising from —C=C—, —C=N—, —NH, —NH_2, —SH, and —N –C=S linkages seem to be absent.[9] Thiazole, thiazoline, and thiazolidine are also excluded by the absence of their specific absorption peaks,[11] and there appears no absorption at 3400 cm.$^{-1}$, which is assigned to the —NH— stretching vibration in pyrrole or indole.[12] Any absorption specific for disulfide linkage is not recognized, as in the case of thioctic acid.[13] These characteristics may suggest the skeleton is a saturated, open-chained, and tertiary amine.

Reduction with Sodium Borohyride ($NaBH_4$)

Nereistoxin smoothly liberated two thiol groups when 50~100 μg. of the hydrogen oxalate were treated with 1 mg. of $NaBH_4$ at *p*H 10 and at 45° C. for one hour, in the manner devised by Nishida[14] for chemical determination of thioctic acid. The resulting thiols were then estimated spectrophotometrically after the method of Boyer,[15] using *p*-chloromercuribenzoate.[14] The formation of 1.9 moles of thiol was observed in the reduction of nereistoxin, and 1.5 moles in thioctic acid, when the estimation of thiol was carried out at *p*H 4.6. At *p*H 7.0, on the contrary, nereistoxin showed a lower value, about 1.6 moles, whereas thioctic acid gave a satisfactory result, as reported by Nishida. The addition compound of reduced nereistoxin with *p*-chloromercuribenzoate revealed an identical absorption spectrum with those of cysteine or dihydrothioctic acid.

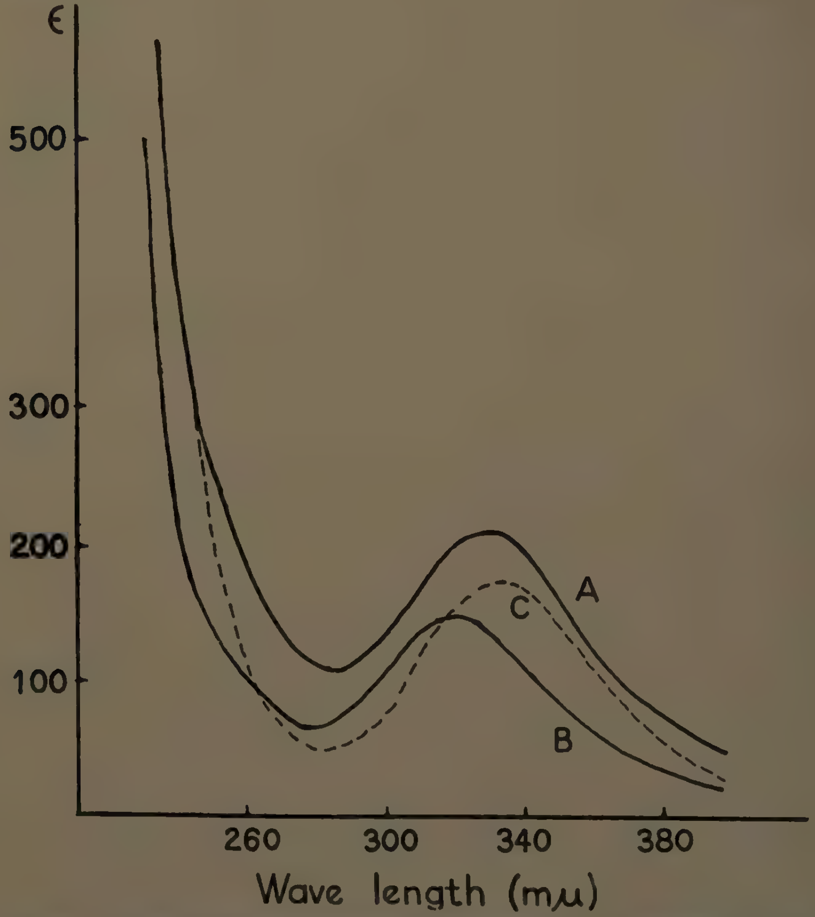


FIGURE 1. Ultraviolet absorption spectra of nereistoxin. A = nereistoxin in 97 per cent EtOH. B = nereistoxin hydrogen oxalate in water. C = 6:8-thioctic acid in EtOH.[8]

Desulfurization with Raney Nickel

Dimethylamine (spot B in FIGURE 3) was recognized as a main degradation product when checked by paper ionophoresis[16] in several trials of desulfurization with Raney nickel, in which nereistoxin was heated at 60∼100° C. for 15∼120 min. with 6∼20 times Raney nickel (W_7) at *p*H 5∼11. A small amount of monomethylamine (spot C) appeared only in prolonged heating at a higher *p*H. The formation of a sulfur-free tertiary polyamine that coincided in mobility with those of five carbons (*N*,*N*-diethylmethylamine; *N*,*N*-dimethyl-*n*-

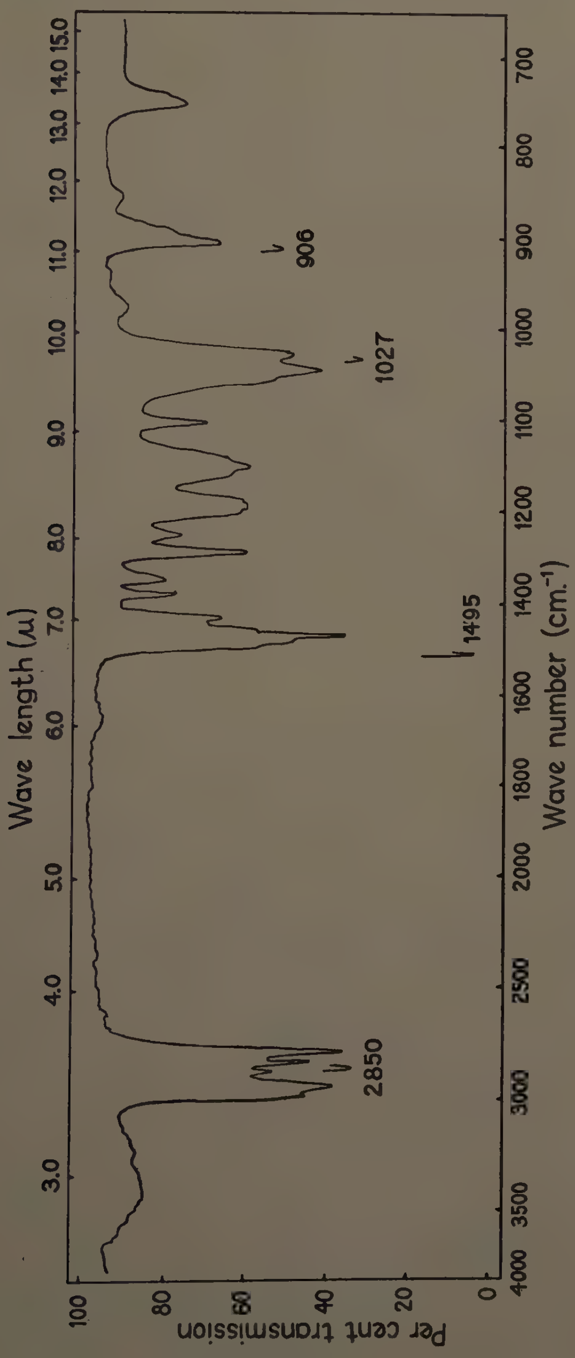


FIGURE 2. Infrared absorption spectrum of nereistoxin in liquid film.

propylamine; or N,N-dimethyl-*iso*-propylamine) was detected only once on the paper chromatogram, but the amine was unobtainable even following several experiments under the same conditions, probably because of instability of that part of the nereistoxin molecule containing sulfur and, also, partly because of the difficulty in preparing Raney nickel of a constant activity. The experimental conditions necessary to obtain this tertiary polyamine are still under investigation.

The mobility of nereistoxin and related compounds in paper ionophoresis is given in FIGURE 3.

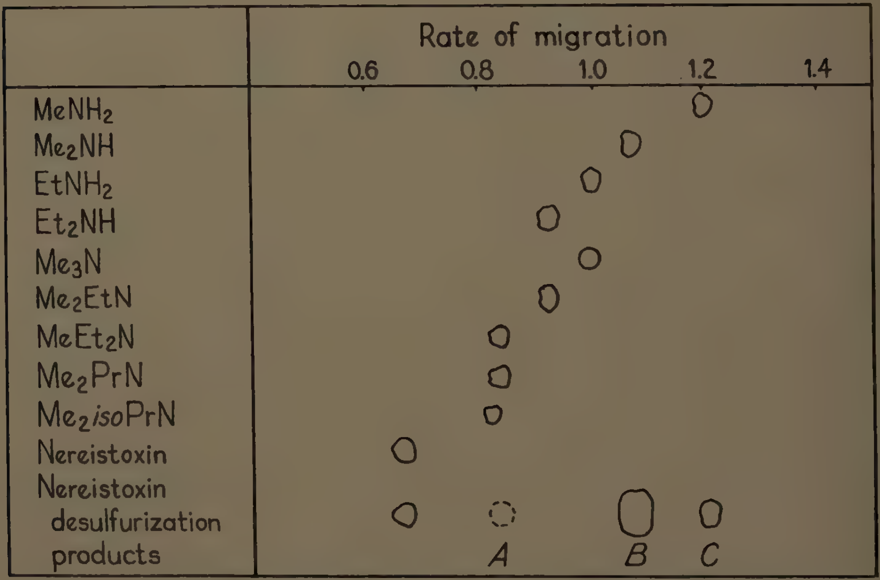


FIGURE 3. Mobility of nereistoxin and related compounds, measured with Toyo Roshi No. 51 filter paper, 12.5 × 36 cm., 11 V/cm., 0.5 ~ 0.75 mA/cm., 0.1 M citrate buffer, according to Weber,[16] pH 3.8, and room temperature of 2° C. for 1.5 ~ 2.5 hours.

Discussion

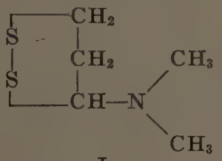
There appeared some disagreements between the results obtained by Nitta and those we found, especially in the melting point of hydrogen oxalate, the odor of the free base, and the empirical formula. The recrystallized hydrogen oxalate showed a constant melting point, which was identical with that of crystals recovered from the filtrate. On the last point, absence of characteristic infrared absorption of —C═C— linkage, and the formation of dimethylamine in desulfurization with Raney nickel, toegther with the negative test for the allyl group, may support our empirical formula, $C_5H_{11}NS_2$, instead of $C_5H_9NS_2$ as given by Nitta.

The positive test for the Dragendorff reagent, infrared absorption spectrum, and other chemical properties indicated the toxin is a tertiary amine. Two atoms of sulfur were clearly shown to be disulfide by color reactions and the liberation of two moles of thiol in reduction with sodium borohydride. Moreover, they were postulated to be cyclic from empirical formulas and ultraviolet absorption spectra.

The similarity of nereistoxin to 6:8-thioctic acid in the ultraviolet absorption pattern is very interesting. With the cyclic disulfides it has been demonstrated that reduction in ring size displaces the absorption band to progressively longer wave lengths and decreases the absorption intensity.[17-19] Thus 4:8-thioctic acid of a seven-membered ring has an absorption peak at 252 mμ (ε 475) as an aliphatic disulfide, a six-membered ring compound, such as 5:8-thioctic acid or tetramethylene disulfide reaches a peak at ca. 295 mμ (ε 300) and a five-membered ring compound, such as 6:8-thioctic acid or trimethylene disulfide has an absorption peak at 332∼334 mμ (ε 150∼160). This may suggest a close resemblance of nereistoxin to 6:8-thioctic acid, in terms of the size of the cyclic disulfide ring.

The occurrence of dimethylamine as a main degradation product in desulfurization with Raney nickel seems to imply that the skeleton of nereistoxin, aside from the cyclic disulfide, is not *N*,*N*-diethylmethylamine, but *N*,*N*-dimethyl-(*n*- or *iso*-)propylamine, although isolation and confirmation of the amine were not attained.

These considerations have led us to postulate one of the following structures (I or II) for nereistoxin, the confirmation of which, however, needs more extensive study and synthesis.



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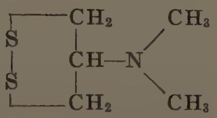

II

Moreover these chemical features suggest that the very interesting and wide-ranged physiological activity of nereistoxin is more than merely neurotoxic.

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SALIVARY POISON OF A MARINE GASTROPOD, *NEPTUNEA ARTHRITICA* BERNARDI, AND THE SEASONAL VARIATION OF ITS TOXICITY

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Consumption of *Neptunea arthritica*, a marine gastropod widely distributed in the northern seas of Japan, has caused many poisonings in Hokkaido. Severe headache, dizziness, and malaises such as seasickness, vomiting, urticaria, and visual disturbances were reported as symptoms of the poisoning. One of us (M. A.) has substantiated the fact that the poison is located only in the salivary gland and not in other organs of the gastropod.[1,2]

Kanna and Hirai[3] reported a similar case of food poisoning caused by the consumption of *Neptunea intersculpta* taken from the Japan Sea area, and the localization of poison only in the salivary gland.

The lethal component of poison (for mice) was localized by conjunctive use of paper chromatography, kymography, and toxicity tests. The lethal component, which reacts strongly positive for the Dragendorff reagent, is very hygroscopic and precipitable with ammonium reineckate or phosphotungstic acid. For the fractionation of the component and its accompanying contaminants (diazo-, dithiocarbamate-, nitroprusside-positive substances), procedures of both Miyaki[4] and Banister[5] were examined and found to be unsatisfactory. Fractionation with an ion exchange resin (Amberlite IRC 50), by Kunin's method,[6] demonstrated the fact that the toxicity is exclusively found in the lysine fraction; however, the method was discarded owing to other disadvantages.

Fractionation by the usual Vickery and Leavenworth method,[7] followed by a toxicity test for each fraction, showed that the lethal component existed only in the lysine fraction, from which it was isolated as picrate and identified as tetramine picrate (TABLE 1).

Further evidence for the identification of lethal component with tetramine was obtained by means of infrared analysis (FIGURES 1, 2, 3), paper chromatography (TABLE 2), and the antagonism experiments.

In a series of antagonism experiments between the salivary poison and several antagonistic reagents (1-adrenaline, eserine sulfate, benadryl, and nicotine sulfate), 1-adrenaline was found to be the most effective for prolonging the survival time after intraperitoneal injection into mice of Red strain (rr). This effect of adrenaline may be ascribed to the relaxation of clonic contraction of the bronchial muscle. The same result was obtained when the authentic tetramine was employed instead of poison in antagonism experiments. Therefore it was confirmed that tetramine is the main pharmacologically-active constituent in the salivary gland.

To determine the predominant substance in the purified concentrate from the salivary gland, prepared after Banister's procedure, we prepared tetraphenylborates according to Marquardt's procedure[8] and perchlorates after Bell's method[9] using the purified concentrate and the known substances (choline, acetylcholine, urocanylcholine, propionylcholine, tetramine, neurine, and trigonelline).

The tetraphenylborates were dissolved in acetonitrile, poured into 0.5-mm. cells, and subjected to infrared absorption analysis with a Perkin-Elmer model 21 infrared spectrophotometer.

The perchlorates were dissolved in Nujol, poured into 0.04-mm. cells, and subjected to infrared analysis. As shown in FIGURES 4 to 7, the spectrum of sample tetraphenylborate coincides with that of tetramine tetraphenylborate within the range of measurement (5 to 7 μ), but the strong band of the CO group near 5.7 to 5.8 μ, which is specific for choline ester derivatives, could not be detected. The spectrum of sample perchlorate also agrees with that of tetramine perchlorate in the entire range of measurements (3 to 15 μ) (FIGURES 8 and 9). Therefore it was proved that the predominant substance in the purified Banister concentrate is tetramine. The spectra of other reference derivatives were dissimilar to those of the sample, and were omitted.

TABLE 1

IDENTIFICATION OF THE LETHAL COMPONENT OF SALIVARY POISON AS TETRAMINE

	Lethal component of salivary poison	Tetramine	Neurine
M.p. of picrate	313° C. (decomp.)*	313° C. (decomp.)	263–264° C.
M.p. of chloroaurate	335–336° C.*	335–336° C.	232–236° C.
Nitrogen content of picrate	Found. 18.48% Subst. 3.062 mg. N = 0.495 ml. 766 mm., 22° C.	Calcd. 18.54%	Calcd. 17.83%

* These derivatives indicate no depression when mixed with authentic tetramine derivatives.

Besides tetramine, the presence of choline compounds and histamine was proved by the following evidence:

(1) Total choline in the gland was estimated microbiologically by Horowitz and Beadle's method,[10] using *Neurospora crassa* mutant ATCC 9277 as the test strain (37.5 mg. per cent). Free choline was only 1.6 mg. per cent.

(2) The purified concentrate obtained by Banister's method showed some decrease of toxicity after being subjected to alkaline hydrolysis (*p*H 12, 100° C., for 10 min.), or after the addition of a small quantity of human blood or mouse blood, suggesting that it is due to the decomposition of choline ester involved.

(3) The total choline content in the hydrolysate of the purified Banister's concentrate was much larger than the ester content of equivalent amounts of the purified concentrate, as measured by Hestrin's method.[11] This implies the coexistence of choline and choline ester.

(4) Histamine in the gland was determined by Tsuda's and Tomiyama's method.[12] Three hundred and sixty gm. of gland contained about 800 γ of histamine (0.22 mg. per cent).

It was Ackermann who first discovered tetramine in the animal kingdom, in

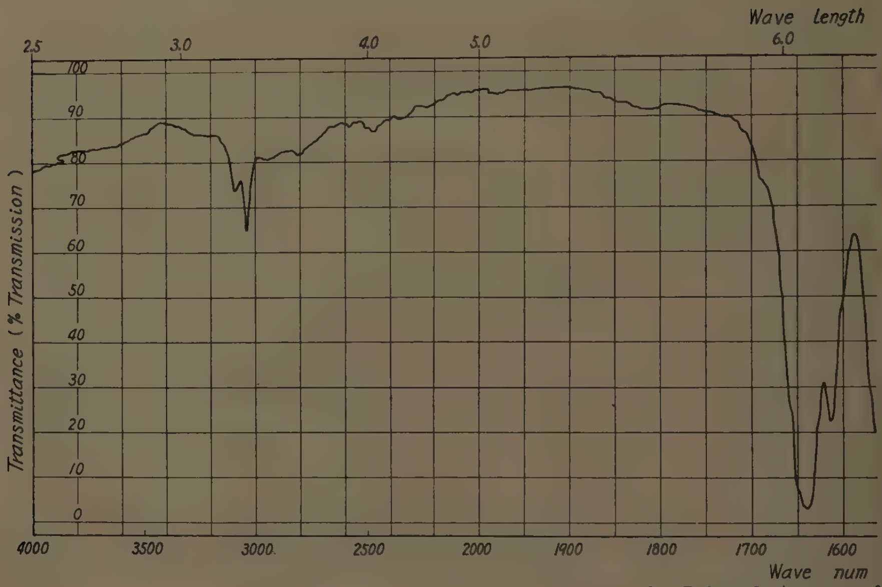


Figure 1. Infrared spectrum of

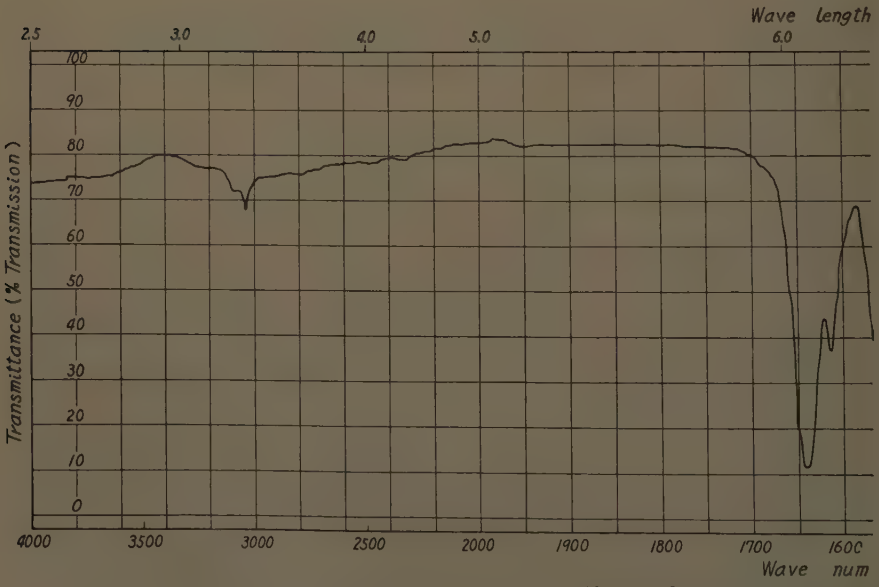
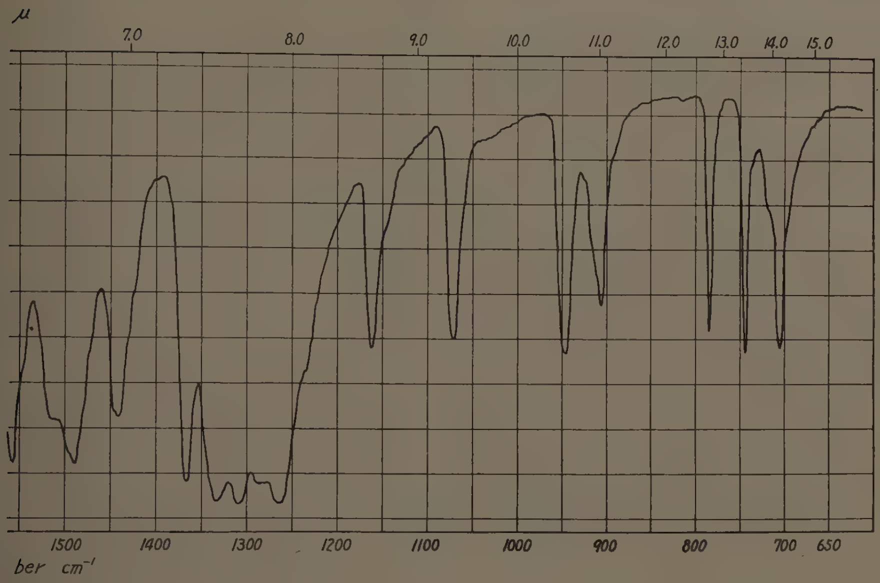
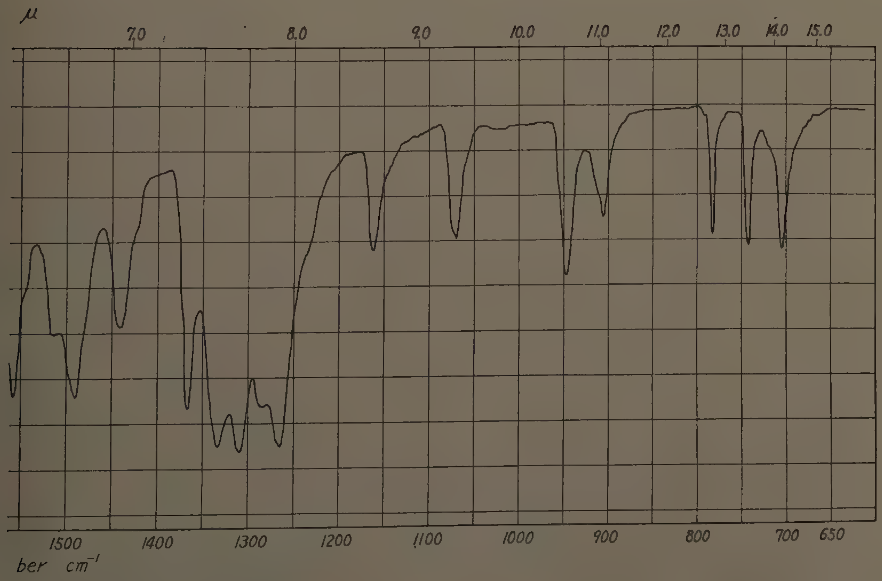


Figure 2. Infrared spectrum



sample picrate (from salivary gland).



of authentic tetramine picrate.

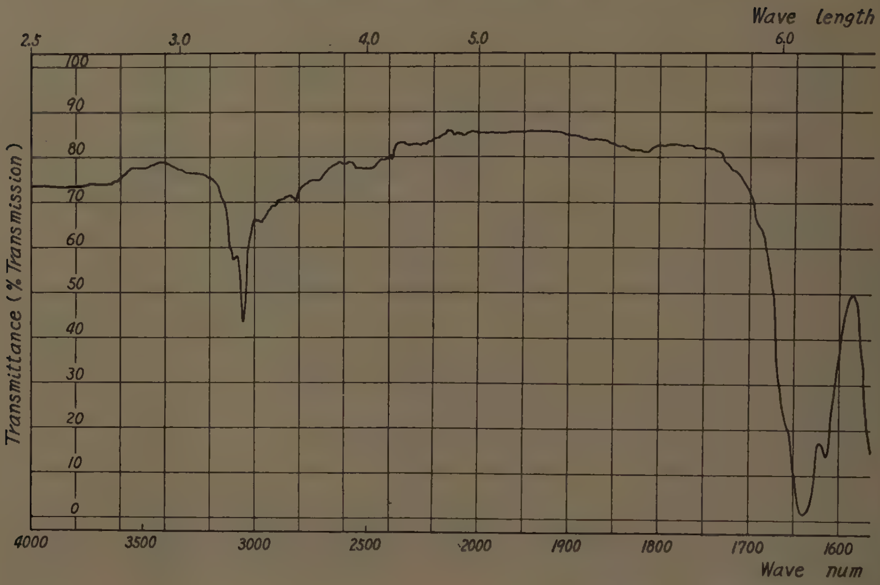


FIGURE 3. Infrared spectrum

TABLE 2

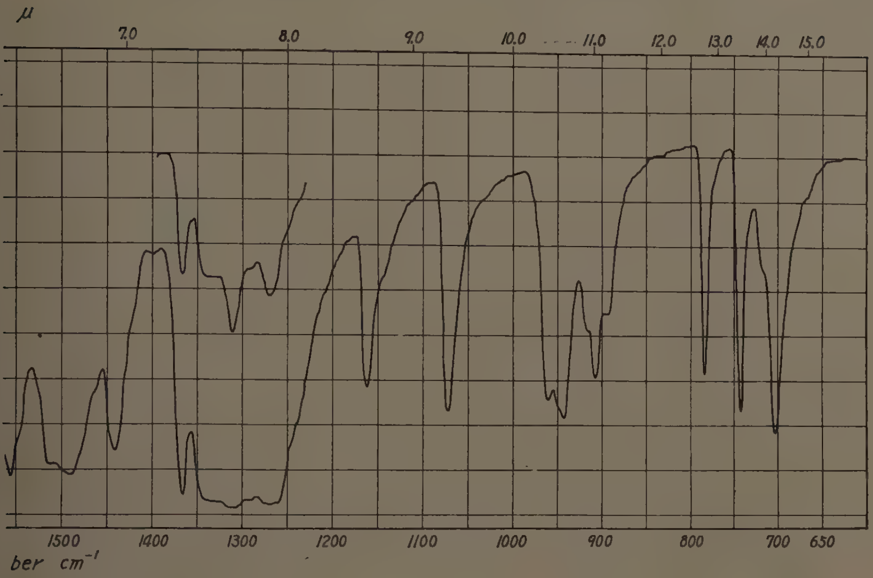
PAPER CHROMATOGRAPHY OF QUATERNARY AMMONIUM BASES AND CHOLINE ESTERS

Spotted substance	R_f values in paper chromatogram run with solvent systems*				
	(1)	(2)	(3)	(4)	(5)
Sample sol. (free form)	0.66, 0.50	0.55, 0.43	0.55, 0.40	0.47, 0.35	0.1–0.3 T†
Sample sol. (HCl salt)	0.37, 0.50	0.25, 0.43	0.23, 0.40	0.15, 0.35	
Tetramine	0.66	0.55	0.55	0.47	0.1–0.3 T†
Neurine	0.69	0.56	0.60	0.47	0.1–0.2
Choline	0.64, 0.49	0.55, 0.45	0.55, 0.35	0.50, 0.35	0.1
Acetyl‡	0.70, 0.52	0.56	0.59	0.50	0.1–0.3 T†
Urocanyl‡	0.27, 0.48	0.52	0.10, 0.48	0.10, 0.47	0.1–0.2
Benzoyl‡	0.86	0.74	0.83	0.75	0.45–0.75 T†
Acetyl-β-methyl‡	0.73	0.64	0.76	0.63	0.1–0.4 T†
Succinyl‡	0.28	0.27	0.10	0.05–0.1	
Myristyl‡		0.9–0.93			0.75–0.90 T†
Lauroyl‡		0.9–0.93			0.80–0.95 T†
β-(3-Indolyl)-propionyl‡	0.84	0.83	0.8–0.85	0.75	0.5–0.65 T†
Trigonelline	0.46	0.33	0.26	0.12	
Nicotine sulfate	0.13, 0.74 T†	0.15–0.20 T† 0.66 T†	0.05, 0.77 T†	0.05, 0.70 T†	

* Solvent systems tested: (1) *n*-PrOH:HCOOH:H_2O (8:1:1); (2) *n*-PrOH:1/N AcOH:H_2O (3:1); (3) *n*-BuOH:AcOH:H_2O (4:1:5); (4) *n*-BuOH:EtOH:H_2O (8:2:1:1); (5) *n*-BuOH saturated with water. Overlapping spots probably were tetramine and choline compounds. Dragendorff and Chargaff-Levine reagents were used as developers.

† T indicates tailing of spot.

‡ Indicates choline esters in each instance.



of authentic neurine picrate.

the coelenterate *Actinia equina*.[13] Recently J. H. Welsh[14] reported the presence of tetramine, homarine, trigonelline, γ-butyrobetaine and dimethylbetaine of imidazole acetic acid in some species of coelenterate determined by paper chromatography and ultraviolet absorption spectroscopy.

Starting with 33.28 kg. of *Actinia*, Ackermann isolated 12.4 gm. of pure tetramine hydrochloride. We obtained about 1.8 gm. of tetramine picrate from 358 gm. of the gland and, later, about 8.0 gm. of picrate from 950 gm. of the gland, a yield of 0.85 per cent. Therefore the tetramine content in the gland was about five or eight times as much as in *Actinia*, when both are compared as picrate.

The seasonal variation of toxicity in the salivary gland was investigated as follows. The shellfish were sampled monthly at Onagawa Bay, Miyagi Prefecture, Japan. The extirpated salivary glands were weighed, homogenized, and extracted twice with ethanol. After distillation of alcohol from the combined extracts, the residue was dissolved in water and defatted by shaking with ether. After removal of ether, the residue was diluted with water to make a solution of 1 gm. gland/ml. water. This original solution was further diluted and 0.5 ml. portions of each dilution were injected into mice to determine the degree of dilution that provided a minimal lethal dose. We regard the smallest amount of poison that kills mice within 30 min. after injection as the minimal lethal dose. The toxicity was expressed as the mouse unit (MU), which is calculated by the following equation:

$$\mathrm{MU} = \frac{\text{volume of original solution (ml.)} \times \text{body weight of mouse (gm.)}}{\begin{array}{l}\text{weight of salivary}\\ \text{gland (gm.)}\end{array} \times \begin{array}{l}\text{volume of injected}\\ \text{solution (usually}\\ \text{0.5 ml.)}\end{array} \times \begin{array}{l}\text{dilution degree of original}\\ \text{solution at minimal lethal}\\ \text{dose } (1/N)\end{array}}$$

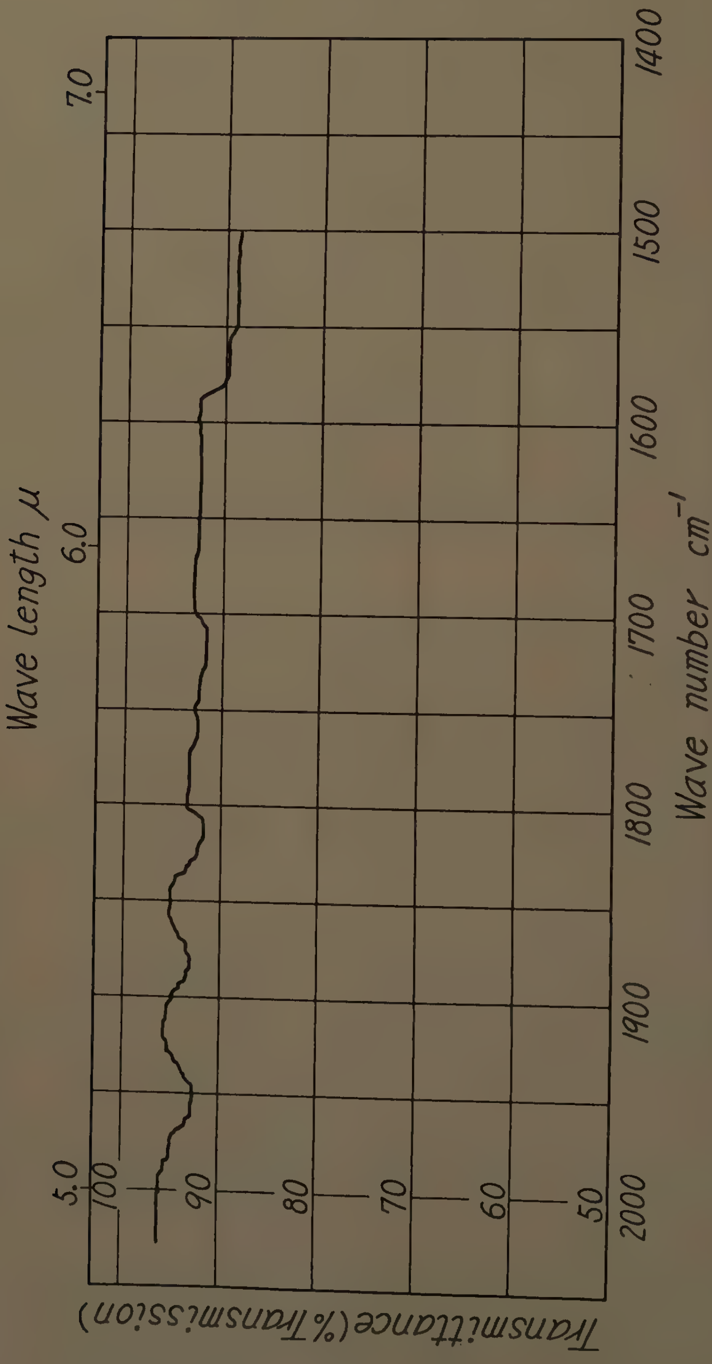


FIGURE 4. Infrared spectrum of sample tetraphenylborate (from salivary gland).

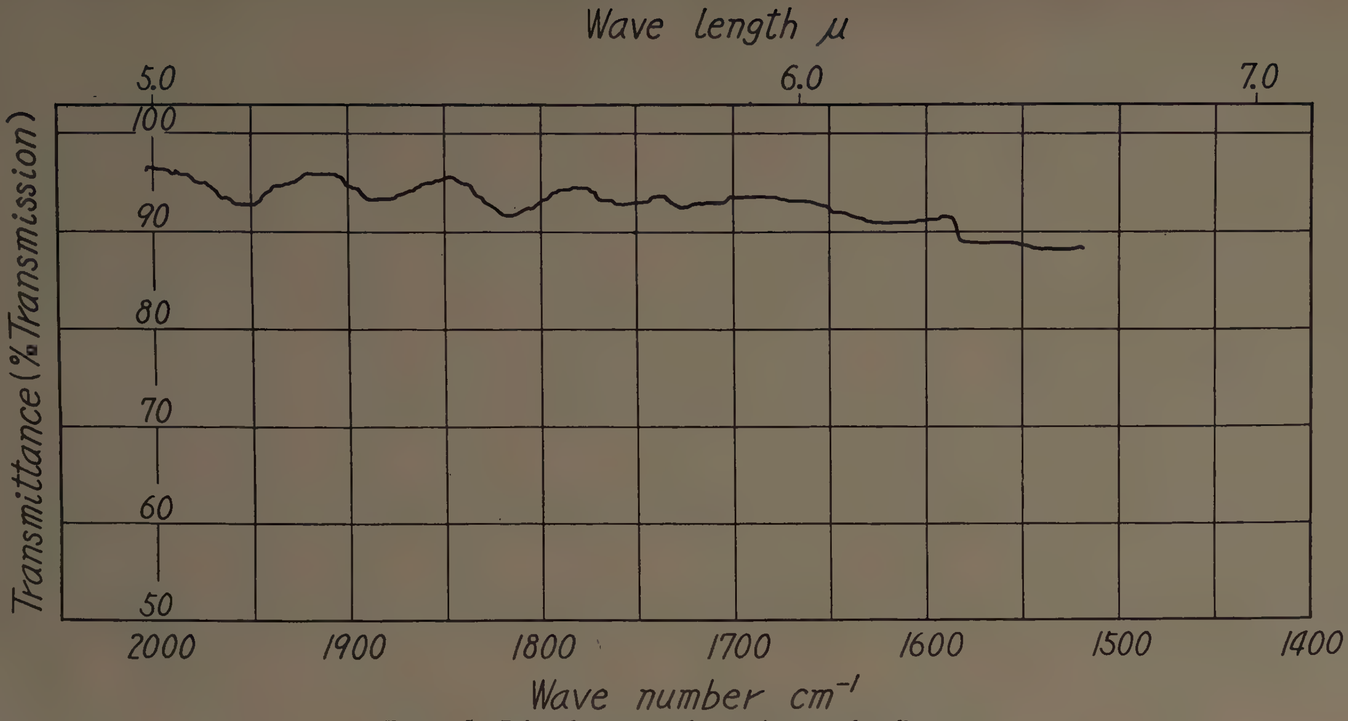


FIGURE 5. Infrared spectrum of tetramine tetraphenylborate.

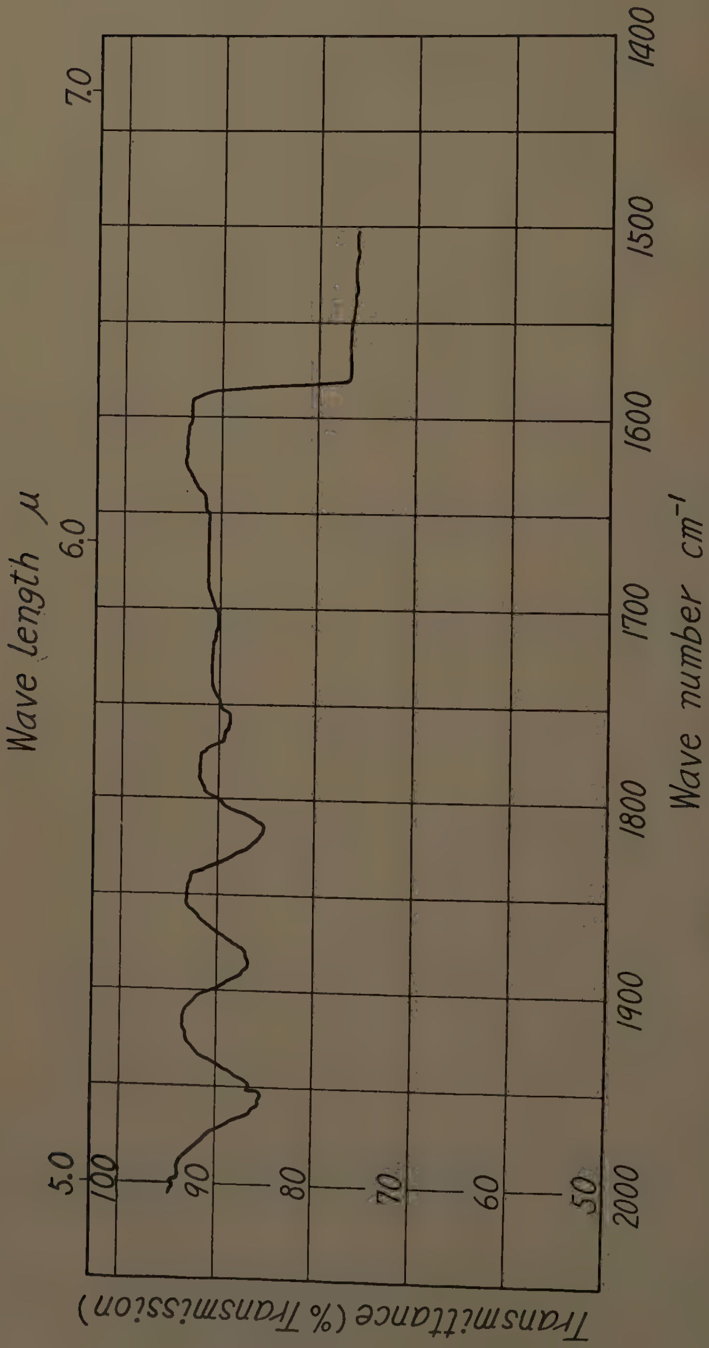


FIGURE 6. Infrared spectrum of choline tetraphenylborate.

The results obtained (TABLE 3) show that the toxicity fluctuates but that the poison's physiological constituents are unrelated to the freshness or post-mortem changes of the shellfish.

Investigation of the toxicity of related species was also carried out. Fresh shellfish were shucked, and each organ (salivary gland, hypobranchial gland, gonad, and mid-gut) was extirpated separately, weighed, and extracted twice with ethanol. After removal of alcohol from the combined extracts, the residue was dissolved in water and made up into 1-ml. and 2-ml. solutions containing the residue from 1 gm. of tissue. This final solution was used for injection experiments. The results of the toxicity test (TABLE 4) proved that the salivary glands of three forms (*Neptunea intersculpta*, *Fusitriton oregonensis*, and *Buccinum leucostoma*) of the 12 species examined are poisonous.

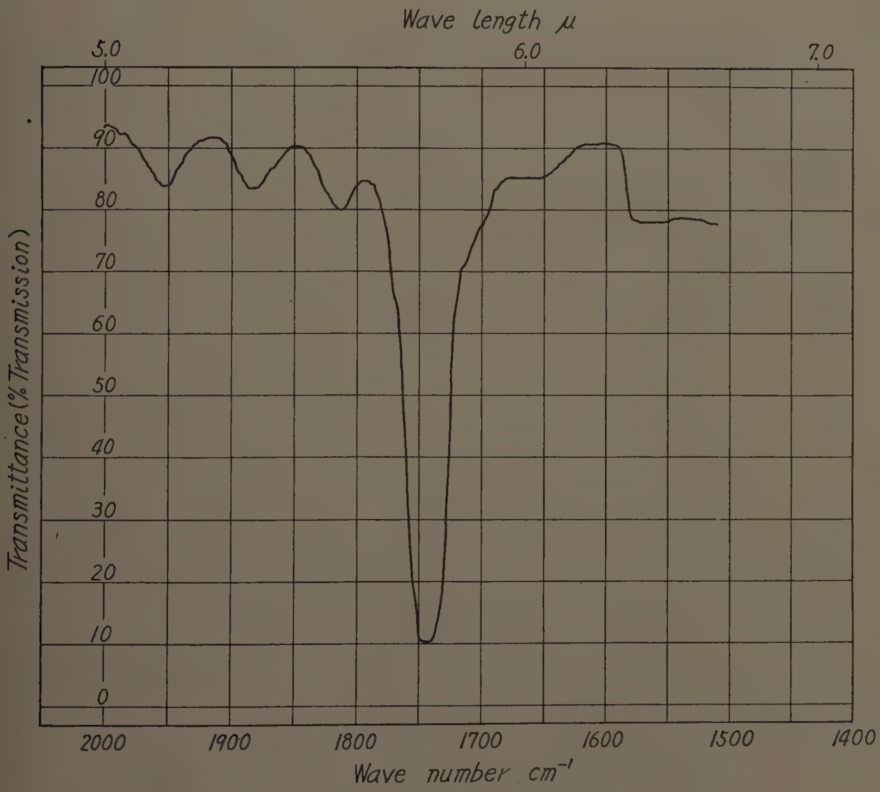


FIGURE 7. Infrared spectrum of acetylcholine tetraphenylborate.

Because we lacked an available determination method for tetramine, we modified the quantitative paper chromatography technique of Miyaki, used for other amines,[15] as follows: the spotted paper strip (7 × 400 mm.) was run with the solvent system of n-PrOH:HCOOH:H_2O, and was developed by spraying it

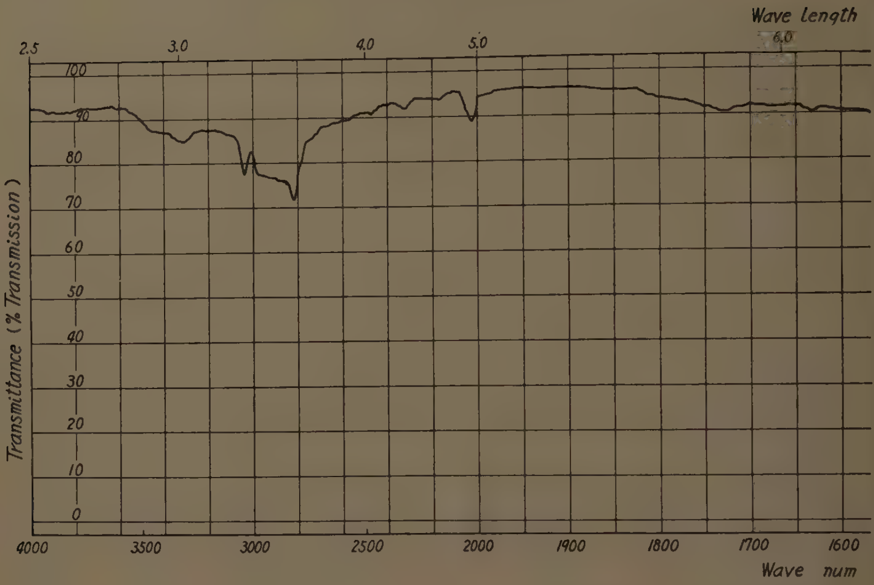


FIGURE 8. Infrared spectrum of

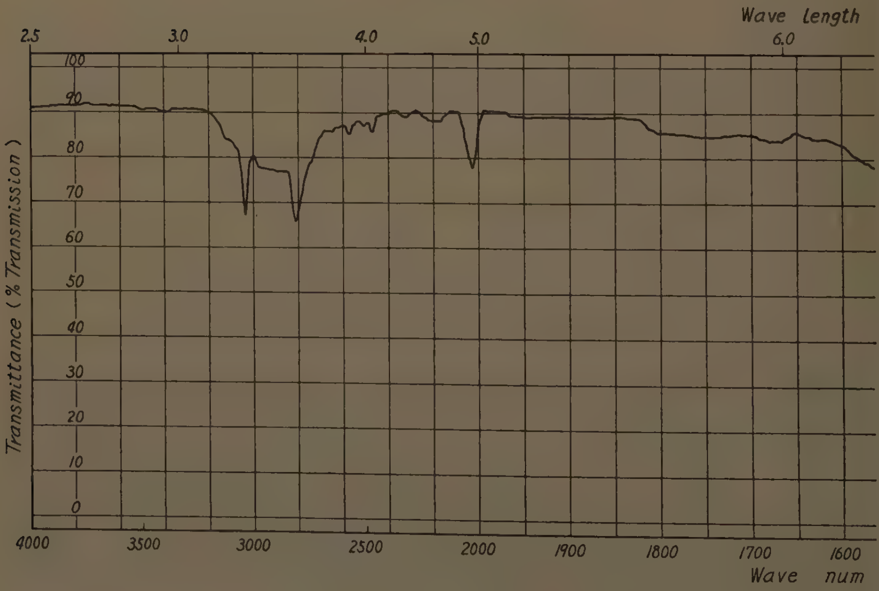
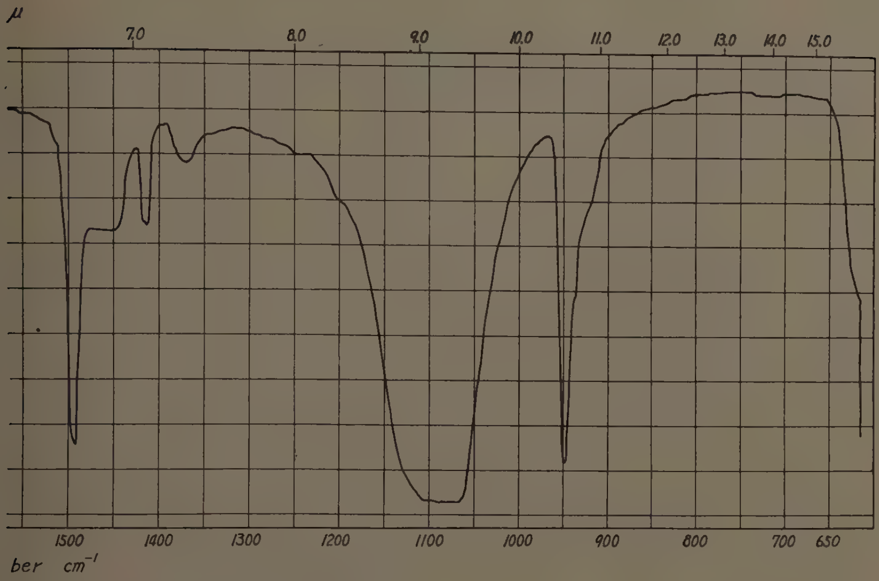
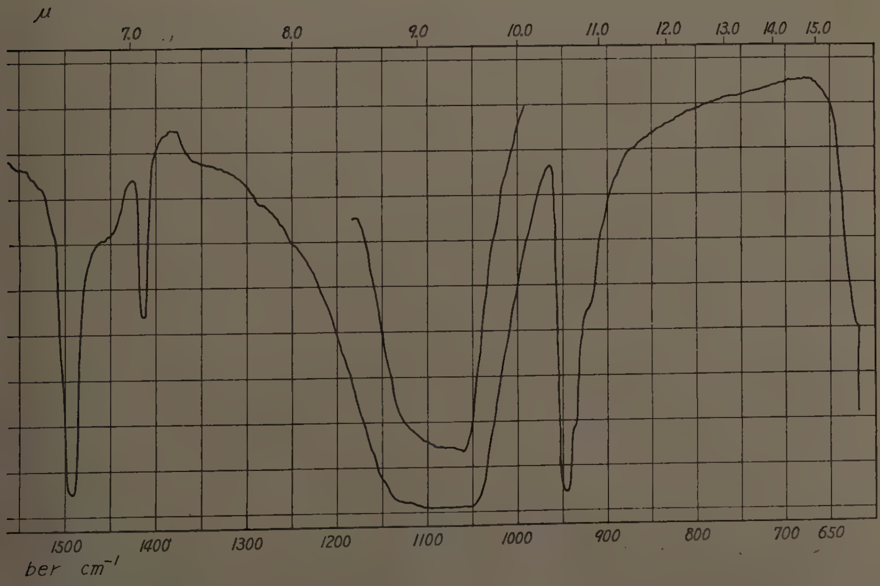


FIGURE 9. Infrared spectrum



sample perchlorate (from salivary gland).



of tetramine perchlorate.

TABLE 3

SEASONAL VARIATION OF TOXICITY IN THE SALIVARY GLAND OF *NEPTUNEA ARTHRITICA*

Date	Mouse unit (MU)
July 7, 1959	384 << 430
August 19, 1959	256
September 20, 1959	240
November 13, 1959	246 << 264
December 14, 1959	280
January 28, 1960	294
February 23, 1960	284

TABLE 4

INVESTIGATION OF TOXICITY OF RELATED SPECIES

Species	Toxicity of tissues*					Injected solution† (site of collection)
	Salivary gland	Hypo-branchial gland	Gonad	Diges-tive gland	Gonad plus digestive gland	
Neptunea arthritica	+	−	−	−		1 gm./1 ml. (Usu)
Neptunea intersculpta	+					1 gm./1 ml. (Mori)
Buccinum leucostoma	+	−	−	−		1 gm./1 ml. (Mori)
Volutharpa perryi	−	−			−	1 gm./1 ml. (Mori)
Babylonia japonica	−	−	−			1 gm./2 ml. (Teradomari)
Fusitriton oregonensis	+	−	−	−		1 gm./1 ml. (Mori)
Rhais clavigera	−	−			−	1 gm./2 ml. (Onagawa)
Thais bronni	−	−			−	1 gm./2 ml. (Onagawa)
Ceratostoma burnetti	−	−			−	1 gm./2 ml. (Onagawa)
Ocenebra sp.	−	−			−	1 gm./2 ml. (Onagawa)
Natica janthostoma	−				−	1 gm./1 ml. (Mori)
Tugalis gigas	−		−			1 gm./2 ml. (Onagawa)

* Key: +, lethal for mouse; −, nonlethal for mouse.

† Extract from 1 gm. of tissue was dissolved in water in the proportion indicated; 0.5 ml. of solution was injected in the animals.

TABLE 5

TETRAMINE CONTENTS OF SALIVARY GLANDS OF *NEPTUNEA* AND RELATED SPECIES

Name	Tetramine content	
	Quantitative paper chromatography	Quantitative isolation
Neptunea arthritica	4.0–7.5 mg./gm. gland	8.4 mg./gm. gland*
Neptunea intersculpta	5.5–9.0	
Fusitriton oregonensis	3.0–4.0†	

* Not recrystallized picrate.

† Developed color differs somewhat from those of the above two species.

with Dragendorff reagent. Tetramine contents of the salivary glands of *Neptunea* and related species were determined by this method (TABLE 5).

The results obtained show that tetramine content of the salivary gland of *Neptunea arthritica* was 7 to 9 mg./gm. of gland at most, while Fänge[16,17,18] stated that the gland of the Swedish species *Neptunea antiqua* contained as much as 20 to 30 mg./gm. of gland.

The pharmacological actions of tetramine consist of curarelike activity and stimulation of the parasympathetic system. This accounts for symptoms such as motor paralysis, respiratory failure (paralysis of bronchial muscle), salivation, lachrymation, miosis, and peristalsis, both in actual food poisoning and in the experimental animals. Tetramine, as the paralyzing reagent of motor nerve ends, resembles tetrodotoxin; however, they are quite dissimilar in chemical nature. The toxicity of tetramine when administered per os to the warm blooded animal is 5 to 10 times weaker than that of tetramine by parenteral injection. Its secretion from the warm blooded animal is very rapid; this accounts for the transient symptoms of the food poisoning.

T. Misawa, in his extensive studies on food allergy,[19] named choline, histamine, and neurine as so-called pseudoallergens. We report here an actual case of food poisoning, chiefly due to tetramine, and wish to regard the compound as one of such "pseudoallergens." Tetramine is a major salivary poison, not only in its quantity but also in its pharmacological activity; however, the synergic effect of histamine and choline compounds must not be overlooked.

Toxicity tests of salivary poison and of authentic tetramine for fish (*Cyprinus carpio*) were carried out by submuscular injection, or by per os administration using a polyethylene catheter. After injection or administration, the following symptoms were observed in both cases: gradual loss of balance, inversion of the abdomen, delayed respiration rate, clonical convulsion and, finally, death. In addition Vallejo-Freire[20] has reported the utilization of quaternary ammonium bases as molluscacides (*Australorbis* sp. snails).

Neptunea is a scavenger feeding on dead fish and crabs. Fishermen catch the shellfish by placing fish carcasses in net baskets and submerging them overnight in the sea.

Tetramine is said to be identical with the so-called thalassin in the tentacle of the sea anemone (*Anemone sulcata*).[21] And J. H. Welsh suggested that tetramine, in conjunction with a specific protein, might be responsible for the paralyzing action of nematocysts. Whether this is true for the gastropod is an unanswered question.

The physiological role of tetramine in the salivary gland of the carnivorous gastropod is not yet elucidated, but its occurrence in marine organisms presents a very interesting problem.

Summary

Tetramine is the major component of salivary poison of a marine gastropod, *Neptunea arthritica* Bernardi. Histamine, choline, and probably choline ester coexist besides tetramine and act as synergists. The poison is contained in the salivary gland throughout the year; removal of the gland, therefore, can protect consumers from food poisoning.

Acknowledgment

We express our gratitude to Y. Tsuchiya for his criticism.

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THE SALIVARY GLAND OF *NEPTUNEA ANTIQUA*[*]

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The largest gastropods occurring in Scandinavian waters are *Buccinum undatum* and *Neptunea antiqua.* The former species has been made the object of several experimental studies and its anatomy has been treated in a monograph by Dakin (1912). *N. antiqua* (FIGURE 1) is the less common of the two species and its anatomy, physiology, and ecology are largely unknown. Only a few data concerning this gastropod are found scattered in the literature. Jeffrey (1867) gives the following information: "This is a good bait for codfish, and a favorite delicacy of the lower working classes in London. At Billingsgate it is sold under the name of 'almond' or 'red whelk'; according to Rutty's history of Dublin the Irish call it 'barnagh,' the tail (liver) being said to be more fat and tender than a lobster." No disadvantages due to enjoyment of "barnaghs," seem to have been reported (this is somewhat astonishing in view of our present knowledge of the poison gland).

In 1956, at the Swedish Marine Biological Laboratory in Kristineberg, I was trying to demonstrate acetylcholine in organs of *Buccinum* by bioassays. Among the *Buccinum* whelks there was also a small specimen, weighing about 25 gm., of *N. antiqua.* An extract of the salivary gland of this gastropod had enormous muscle-contracting activity. Similar results were obtained in experiments on salivary gland extracts from other larger specimens. The physiological effect was not due to acetylcholine (Fänge, 1957). Further studies seemed to indicate the presence in the salivary gland of *Neptunea* of neurine or some other quaternary ammonium compound (Fänge, 1958; Emmelin and Fänge, 1958).

When the work on *Neptunea* was started I was not aware that Asano in 1952 had described cases of food poisoning in Hokkaido, Japan, caused by ingestion of *Neptunea arthritica.* Asano's investigation showed that the poison was located in the salivary gland. Recently Asano and Itoh (1959) have published a careful biochemical and pharmacological study identifying the salivary poison as tetramine. Similar poison was found in glands of three other gastropod species.

The present article reports some observations made on the salivary gland of *N. antiqua.* The material for these studies was obtained from the Kristineberg Zoological Station on the Swedish west coast.

General Appearance of the Gland

In relation to the size of the snail the gland is a large organ. The weight of the gland comprises 3 to 5 per cent of the body weight (without shell).

The gland consists of two lobulated yellowish-white masses situated on each side of the oesophagus at the level of the brain ganglia (FIGURE 2). From the gland tissue on each side a long secretory duct runs in a forward direction later-

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ally to the oesophagus. Dorsally the surface of the gland is slightly convex and rather smooth. Ventrally the gland stretches into niches between bundles of body wall muscles.

In the living condition the gland has a loose consistency and may easily be torn into pieces. An opaque fluid with a fishy smell may then be squeezed out of the damaged tissue. The *p*H of such fluid has been measured with indicators and was 7.0.

Microscopic Structure

A number of histological preparations recently made by N.-P. Berg Justesen of the Zoophysiological Institute in Oslo, Norway, have revealed interesting details of the cytology of the gland.

In paraffin sections made by ordinary histological methods the gland tissue appears filled with numerous secretory granules or vacuoles. These are 4 to 10 μ in diameter and are brightly stained. Irregular membranes probably representing damaged cell membranes are seen, but there are no distinct cell nuclei. The tissue seems to be badly damaged by Bouin's fluid (FIGURE 3, *right*).

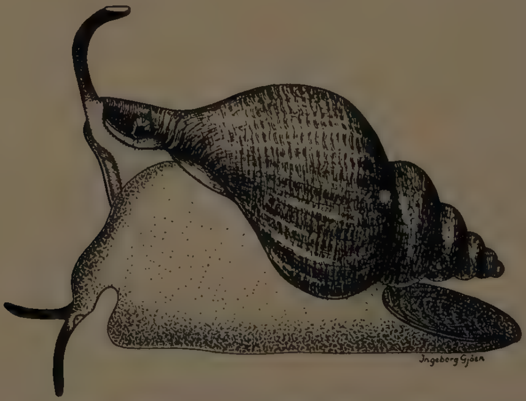

FIGURE 1. *Neptunea antiqua.* Living specimen. One third natural size.

In unstained freshly cut frozen sections there are no large secretory granules. Instead, the tissue contains small uniform granules with a diameter of about 1 μ. If such frozen cut sections are air-dried, fixed in abs. ethanol, and afterwards briefly treated with a solution of thionin in 90 per cent ethanol, the granules swell a little and stain metachromatically. At the same time a number of cell nuclei are stained blue by the thionin (FIGURE 3, *left*).

As far as may be judged from these microscopic studies the gland is chiefly composed of cells measuring approximately 20 μ in diameter and containing the above-mentioned cytoplasmic granules. In addition there are at least two other less abundant types of cells, one of which shows enterochromaffin reactions.

In the sections are seen a number of ducts containing smooth muscle fibers in their walls and seemingly lined with a ciliated epithelium.

The Presence of Tetramine

Paper electrophoresis. Extracts of salivary glands of *N. antiqua*, made by crushing the glands in ethanol or distilled water, were studied by paper electrophoresis. As substances for comparison choline, neurine, and tetramine were used. The pH was 6.5 in a solution consisting of pyridine-acetic acid-

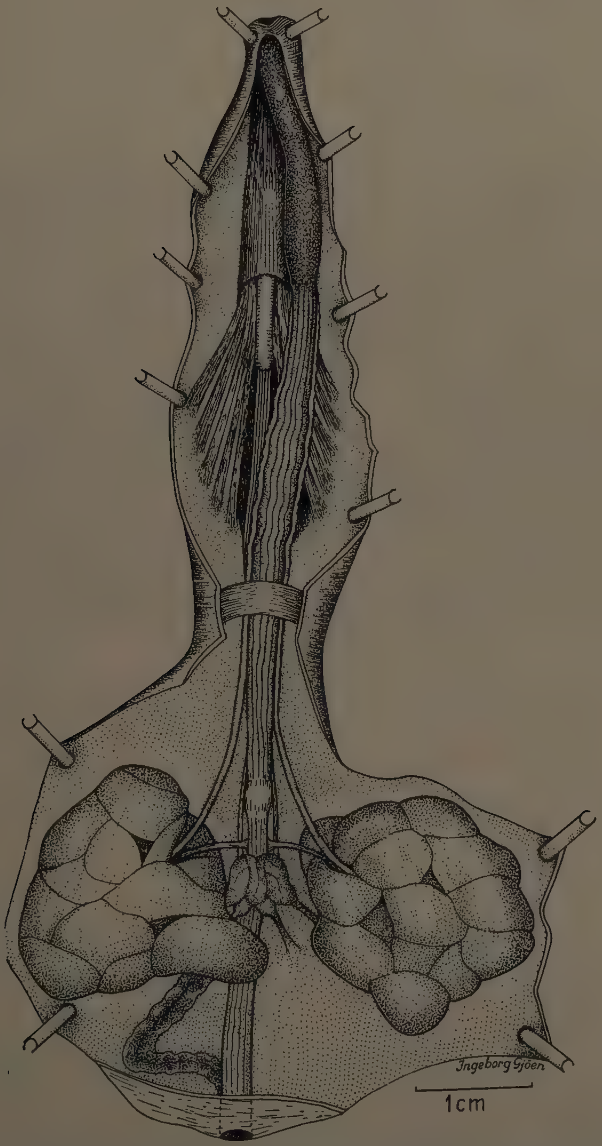

Figure 2. Dissection preparation of the salivary glands and adjacent structures. Between the two gland masses a part of the central nervous system is visible. The structure underlying the anterior part of the oesophagus is the myoglobin, containing radula muscle. Dorsal view.

water in the proportions 90:10:900. The electrophoreses were run for 3 hours at 500 v. After drying, the papers were sprayed with dipicrylamine solution, by which the reagents choline, neurine, and tetramine are stained red, while choline esters usually appear as brown spots (Ackermann and Mauer, 1943; Augustinsson and Grahn, 1953).

The results of two electrophoretic experiments are seen in FIGURE 4. The extracts always produced a very large red spot probably caused by tetramine. In addition to this dominant spot two to four weak spots caused by other substances were observed. One of these might have been acetylcholine and another choline.

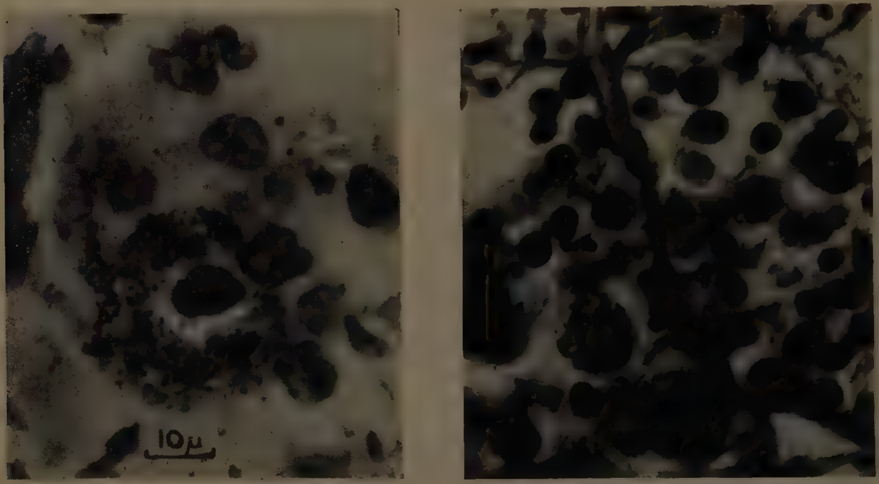

FIGURE 3. *Neptunea antiqua*, salivary gland. Microphotographs. To the left: air-dried frozen section fixed in 100 per cent ethanol and stained with thionin in 90 per cent ethanol. Nuclei and small granules are seen. To the right: paraffin section stained with mallory. The gland was fixed in Bouin's fluid. No distinct nuclei are seen but many large, brightly stained granules or vacuoles are visible. Magnification was the same in both pictures.

The concentration of tetramine in the gland. By paper chromatography and paper electrophoresis of the *Neptunea* salivary gland extracts, only minute amounts of other amines are found in addition to tetramine. Furthermore, extracts of the gland do not lose activity when treated with cholinesterase or by boiling at an alkaline *p*H (Fänge, 1957). Thus tetramine is probably responsible for close to 100 per cent of the physiological activity of the extracts.

An attempt was made to estimate the content of tetramine in a gland by bioassay. Using the isolated *Buccinum* radula muscle as a test object, it was found that almost 1 per cent of the gland seems to consist of tetramine (9 mg./gm. of gland tissue). This figure is, of course, approximate and might be a little too high. Asano and Itoh (1959) found by quantitative paper chromatography that the salivary gland of *N. arthritica* contained 2 to 7.5 mg. of tetramine per gm. of gland tissue. The agreement between the figures is rather good.

Observations on Living Specimens

In aquariums, *Neptunea* does not take food as readily as *Buccinum*, which indicates that it is more specialized in its feeding habits. In nature, *Neptunea* presumably uses its poison to attack other animals, on which it feeds. No such attacks have been observed in aquarium conditions, but the snails often accept dead crustaceans such as *Carcinus maenas* or *Nephrops norwegicus* as food. The intestinal content of snails newly caught usually is red, indicating that their natural food consists of red-pigmented invertebrates.

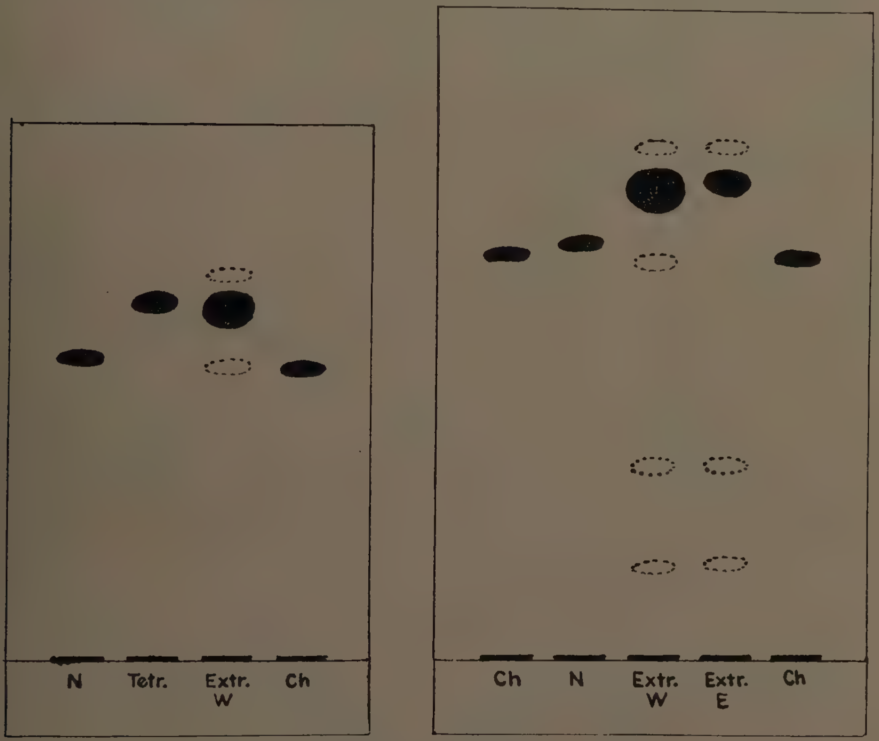

FIGURE 4. Drawings showing the results of two paper electrophoretic experiments. Abbreviations: N, Neurine bromide; Tetr., Tetramine chloride; Ch, Choline chloride; Extr. W, extract made with water; Extr. E, extract made with ethanol. After electrophoresis the filter papers were sprayed with a solution of dipicrylamine. Black spots indicate red color with dipicrylamine. Dotted outlined areas indicate weak spots produced by unidentified compounds in the extracts. Paper electrophoresis by P. H. List.

In small specimens of *Neptunea*, the salivary poison might be of value not only for the capture of prey, but also for defensive purposes.

The way in which the snail gives off the poison is not known. When the shell of a living snail is crushed, the animal stretches out its proboscis and a small amount of fluid appears in the mouth. A similar reaction of the proboscis is known from other carnivorous gastropods such as *Dolium* and *Cassidaria* from the Mediterranean Sea, but these later species are also able to eject repeated jets of secretion from the salivary gland. *Neptunea* probably lacks the muscular apparatus necessary for rapid ejection of saliva and, instead, releases the saliva in repeated small portions into the surrounding sea water.

Discussion

Although no analyses have been made of the fluid secreted by the gland, tetramine undoubtedly is a main secretory product. Because the fluid coming out from a cut gland is neutral, the tetramine must be present as a salt with a strong acid.

Before it is secreted the tetramine probably is bound to some structures in the cytoplasm of the gland cells. The small labile granules observed in fresh-frozen sections might be suspected to contain tetramine. The metachromatic stain property displayed by these granules might indicate that they are partly composed of organic sulfuric esters.

Acknowledgments

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PHARMACOLOGICALLY ACTIVE CHOLINE ESTERS IN MARINE GASTROPODS

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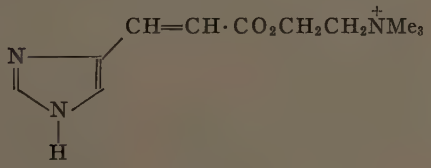
Introduction

The hypobranchial glands of marine prosobranch gastropods of the family Muricidae have attracted the interest of chemists as the site of formation of a chromogen which, in the presence of light and air, is rapidly oxidized to an intense purple dye. This dye was well known to the ancient world under the name Tyrian purple, and vast deposits of the crushed shells of various species of Murex have been found at Tyre and other centers of the dye industry in the Mediterranean. Friedländer[1] showed that the dye is 6:6′-dibromoindigo; Bouchilloux and Roche[2] have also detected the unbrominated chromogen in certain species.

Pharmacological action by extracts of the gland was discovered by Dubois, whose valuable monograph[3] describes their toxic and paralyzing action in both warm- and cold-blooded species. The pharmacology of the glandular extracts was further studied by Roaf[4] and Vincent and Jullien,[5] who showed that they contained a high concentration of an acetylcholinelike substance. The findings of Dubois and Vincent and Jullien were clarified and related by the isolation from the glands by Erspamer and his co-workers[6,7] of a new tissue base, murexine or urocanylcholine (cation as in I), which combines the ganglion-stimulating properties of acetylcholine with a strong neuromuscular blocking action.[8]



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Meanwhile, Whittaker and his co-workers[9,10] had developed sensitive paper and column chromatographic methods for separating and identifying choline esters in connection with their studies of choline esters in mammalian tissues, which resulted in the identification of propionylcholine in ox spleen.[10,11] In 1954 it was decided to carry out a survey of marine gastropods, and the species listed under reference 12 in TABLE 1 were examined. All the Muricidae were found to have high acetylcholine equivalents when extracts of their hypobranchial glands, or of whole organisms, were tested on the frog rectus abdominis muscle. Interestingly, *Buccinum undatum* which, although not a member of the family Muricidae and devoid of chromogen in its hypobranchial gland, is nevertheless closely related, also had a high acetylcholine equivalence, but *Busycon canaliculatum*, another Rachiglossa, had no activity. The noncarnivorous, alga-eating Taenioglossa *Littorina littorea* had little or no activity.

Studies[12] with synthetic urocanylcholine and other esters showed that they were readily separated by chromatography on columns of weak acid ion-exchange resins from each other and from homarine, a tissue base present in relatively high concentration in molluscan tissues but devoid of pharmacological effects on the frog rectus preparation. Homarine, as a zwitter ion (II) has little or no affinity for the resin and is readily washed through with water or buffers. Acetylcholine and its homologues, as univalent cations, combine with the resin, but are eluted at *p*H 4 to 4.5, the quantity of buffer required

TABLE 1
SPECIES OF INVERTEBRATES EXAMINED FOR PHARMACOLOGICALLY ACTIVE ESTERS OF CHOLINE

Family	Species	Tissue*	Ester present	Reference
Mollusca: Gastropoda				
Prosobranchiata				
Rachiglossa				
Muricidae	*Murex trunculus*	HG	urocanylcholine	6,7
	M. brandaris	HG	urocanylcholine	6
	M. fulvescens	HG	urocanylcholine	12
	Tritonalia erinacea	HG	urocanylcholine	6
	Urosalpinx cinereus	WO	urocanylcholine	12
	Thais lapillus	HG	urocanylcholine	12
	T. floridana	HG	senecioylcholine	12
Buccinidae	*Buccinum undatum*	HG	acrylylcholine	12
Turbinellidae	*Busycon canaliculatum*	HG	none	12
Taenioglossa				
Littorinidae	*Littorina littoria*	WO	none	12
Cerithiidae	*Vulgocerithium vulgatum*	WO	none	6
Turritellidae	*Turritella communis*	WO	none	6
Pelecypoda				
Teleodesmacea				
Veneridae	*Venus mercenaria*	WO	none	12
Mytilidae	*Mytilus edulis*	HP	none	6
Arthropoda: Crustacea				
Decapoda				
Homaridae	*Homarus americanus*	NC	acetylcholine	12
Insecta				
Lepidoptera				
Arctiidae	*Arctia caja*	CG	senecioylcholine	22

* Tissues: HG, hypobranchial gland; WO, whole organism; HP, hepatopancreas; NC, ventral nerve cord and limb nerves; CG, cervical gland. The entry "none" does not exclude small but possibly functionally important amounts of choline esters in, for example, nervous tissue.

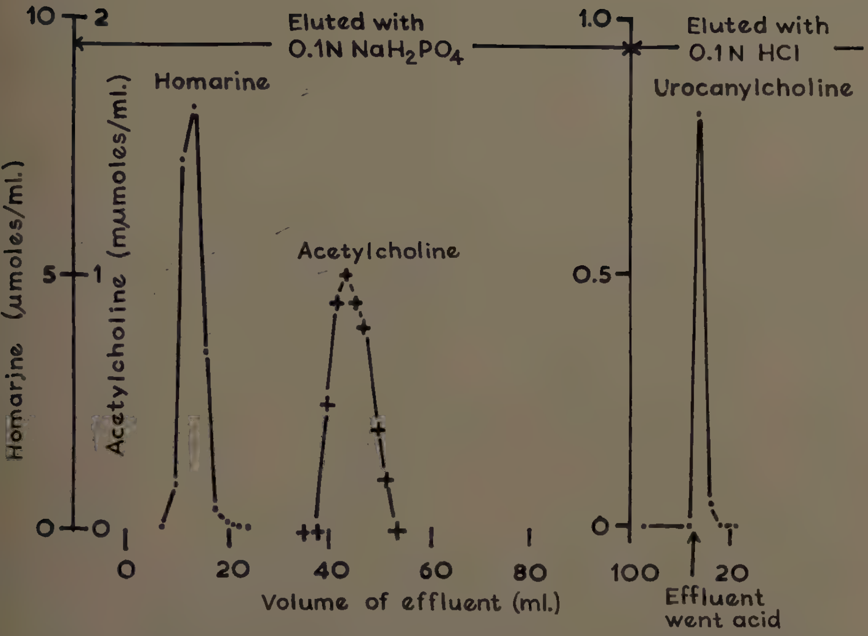


FIGURE 1. Chromatographic separation of synthetic homarine (44 μmoles, 93 per cent recovery), acetylcholine (0.015 μmole, 70 per cent recovery) and urocanylcholine (1.8 μmoles, 95 per cent recovery) on a column of the weak acid ion-exchange resin, Amberlite XE-97. Homarine and urocanylcholine were estimated spectroscopically, acetylcholine by frog-rectus assay.

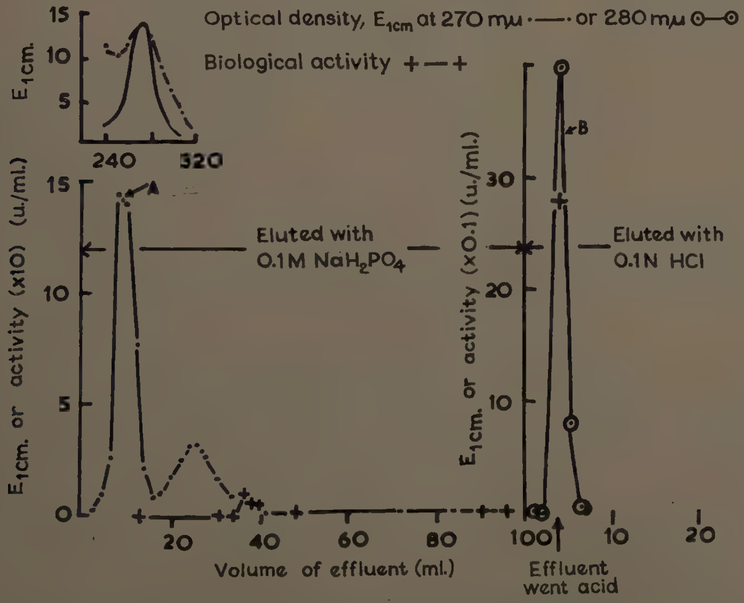
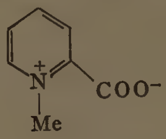


FIGURE 2. The chromatographic identification of homarine and urocanylcholine in a hypobranchial extract of *M. fulvescens*. Peak A was identified as homarine by its retention volume and its absorption maximum (*inset*) at 270 mμ; urocanylcholine (peak B) by its chromatographic behavior, biological activity, and absorption spectrum (see FIGURE 3).



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for elution depending on the chain length of the ester. Choline esters capable of ionizing as divalent cations, such as urocanylcholine, are more strongly held

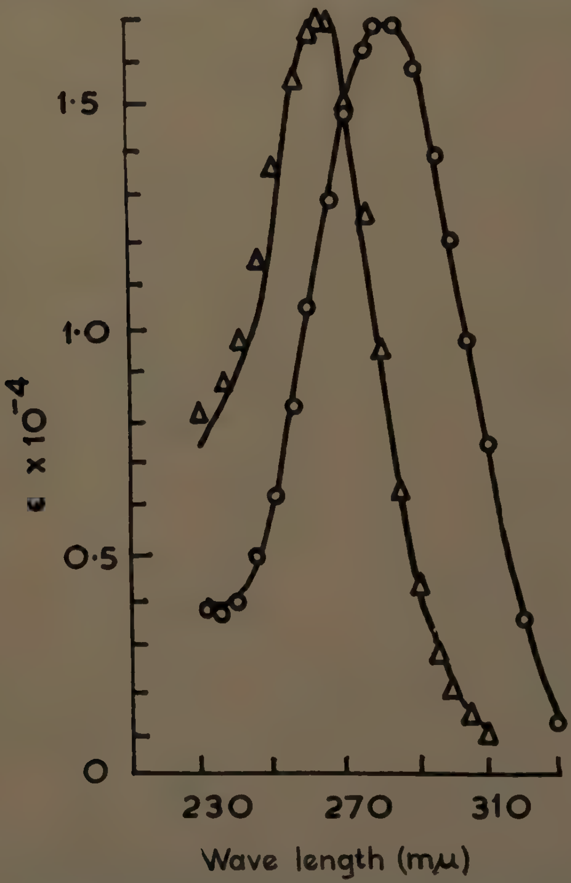


FIGURE 3. Absorption spectra of active ester from *M. fulvescens* (○) and its hydrolysis product (△) at *p*H 4.5, compared with authentic urocanylcholine and urocanic acid (*continuous lines*).

by the resin and require 0.1 *M* HCl for elution. The separation of homarine, acetylcholine, and urocanylcholine is illustrated in FIGURE 1.

Chromatographic Identification of Urocanylcholine

Chromatography of extracts of *Thais lapillus*, *Murex fulvescens*, and *Urosalpinx cinereus* revealed the presence of urocanylcholine as the main active

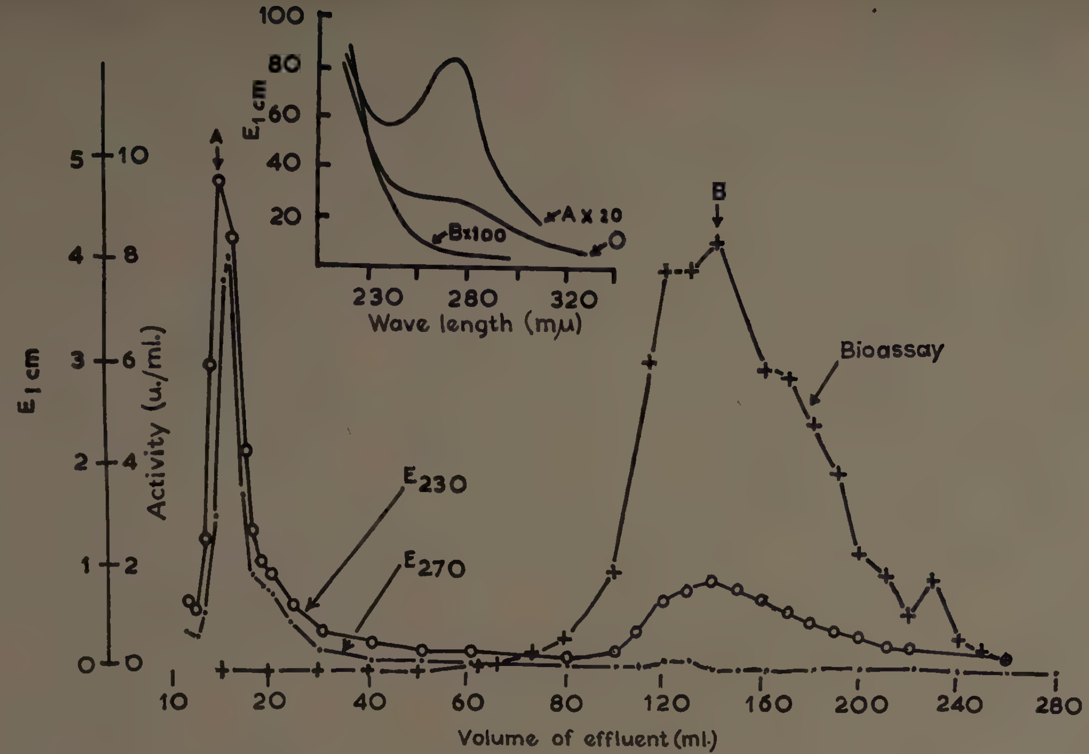


FIGURE 4. Chromatography of active material from *T. floridana.* Eluant, 0.1 *M* NaH_2PO_4. Peak A again has an absorption maximum at 270 mμ (*inset*) and is homarine. The active component (peak B), unlike urocanylcholine, is eluted by phosphate and has an absorption peak (*inset*) below 230 mμ. The absorption spectrum of the original extract (O *in inset*) shows the combined contribution of homarine and the new ester.

component. A typical chromatogram is shown in FIGURE 2; the active material eluted by 0.1 *M* HCl was further identified as urocanylcholine spectroscopically as shown in FIGURE 3. The characteristic shifts in the value of λ_{max} with *p*H were also obtained.

By contrast, the active materials present in extracts from *T. floridana* and *Buccinum undatum* behaved similarly to a univalent choline ester (FIGURES 4, 5) containing 5 to 6 carbon atoms (*T. floridana*), or 2 to 3 carbon atoms (*B. undatum*) in the acyl group.

Identification of Active Material from T. floridana and B. undatum

The material from *T. floridana* was labile in the presence of alkali or human plasma cholinesterase; choline was identified chromatographically in the hydrolysate. UV spectroscopy revealed a peak at 222–223 mμ suggestive of an αβ-double bond. The presence of an ester link was demonstrated by the ferric hydroxamate reaction, the presence of a double bond was confirmed by measurements of bromine uptake, and an unsaturated volatile fatty acid was isolated from hydrolysates by steam distillation. The reduction product of this acid was identified as *iso*valeric acid by vapor-phase chromatography; since senecioic acid is the corresponding αβ-unsaturated fatty acid, the active material from *T. floridana* must be senecioylcholine (III).

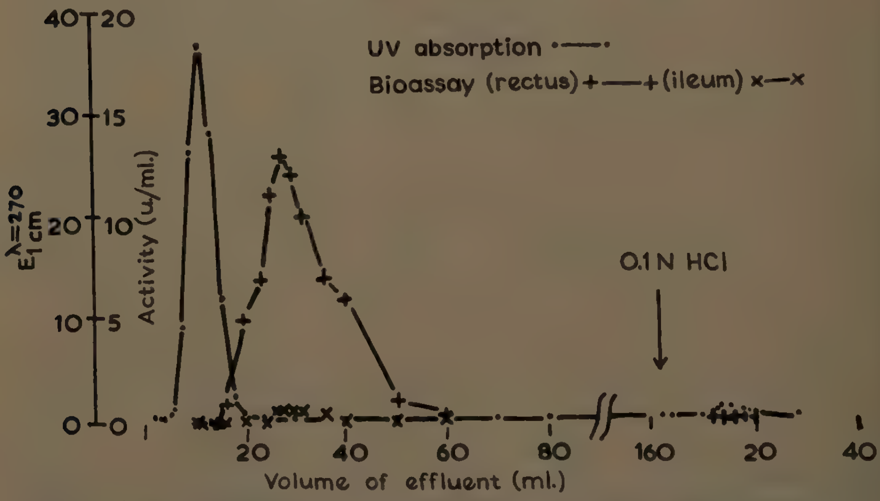
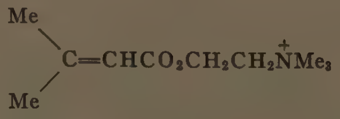


FIGURE 5. Chromatography of active material from *B. undatum*. The active materia has an even lower retention volume than that from *T. floridana*, and is suggestive of a C_3 ester



$$(Me)_2C{=}CHCO_2CH_2CH_2\overset{+}{N}Me_3$$

III

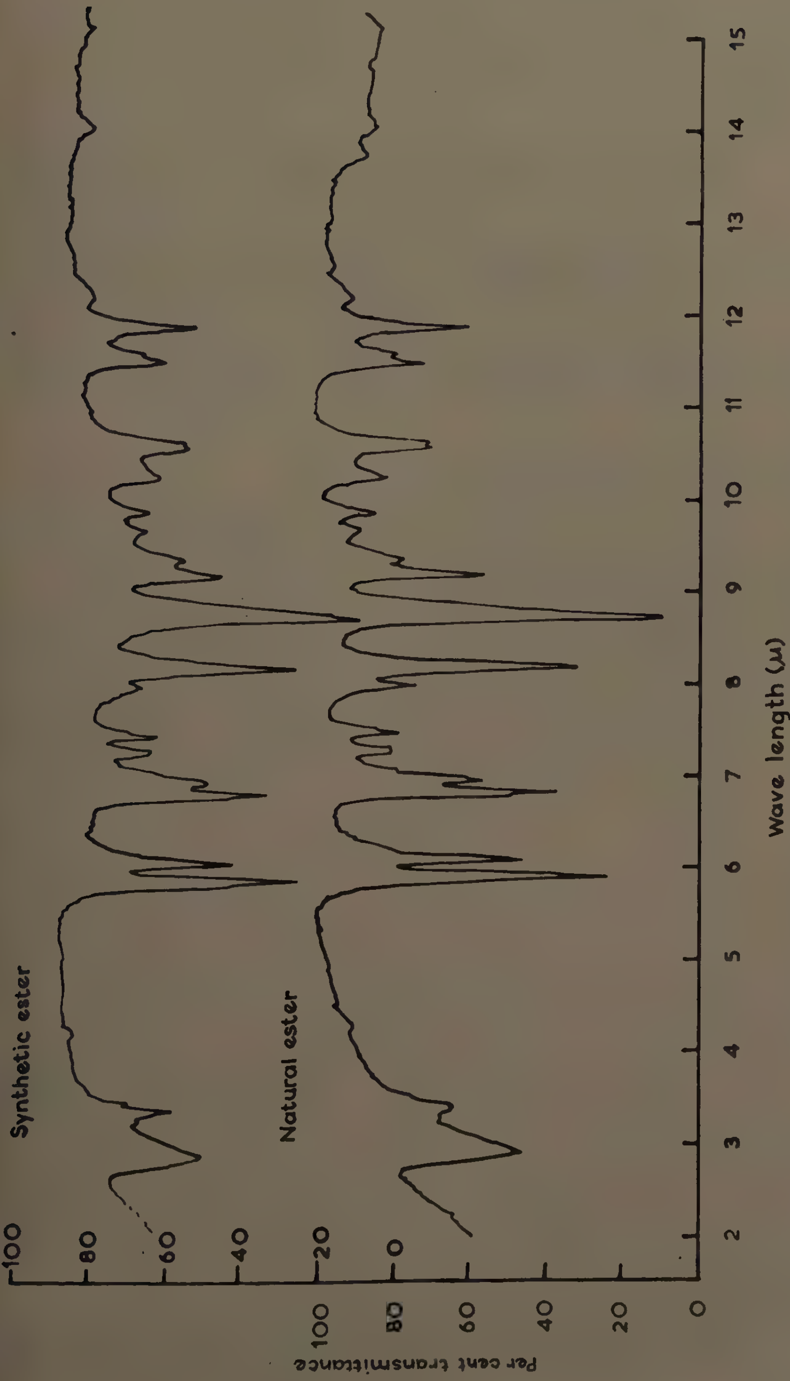


FIGURE 6. Infrared spectrum of natural and synthetic senecioylcholine.

This identification has been confirmed by the synthesis of the compound that is identical with the natural product in pharmacological, chemical, and physical tests including infrared absorption (FIGURE 6).[13]

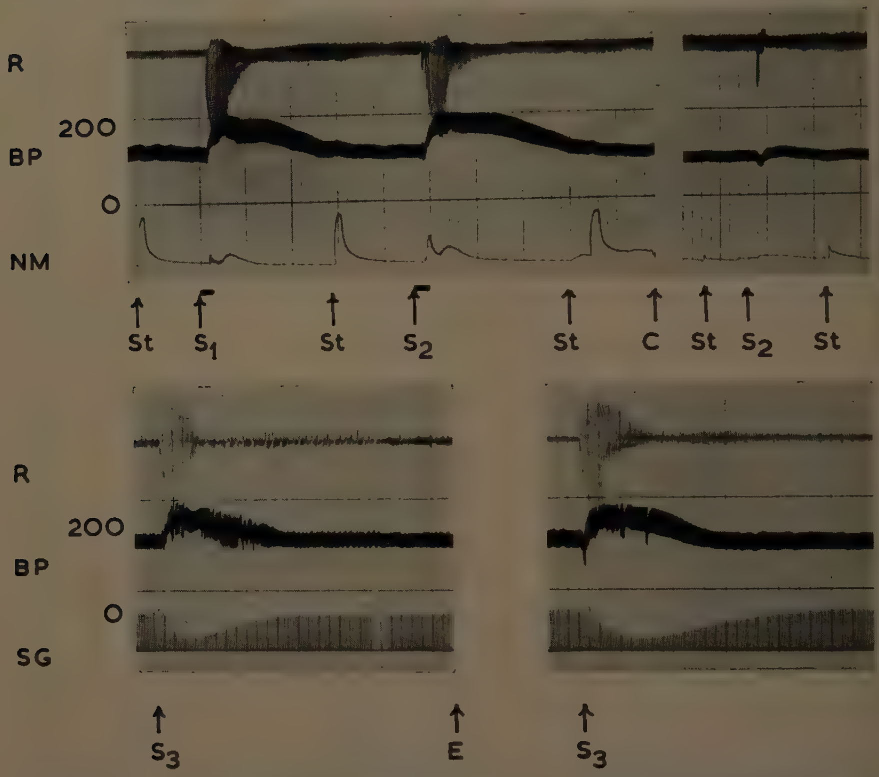


FIGURE 7. *Upper record:* Effect of senecioylcholine on the respiration (R), blood pressure (BP), and nictitating membrane (NM) of the cat. Weight of cat, 4.5 kg.; anesthetic, allobarbitone; time, 10 sec.; pressure in mm. Hg. S_1, S_2, injections of senecioylcholine bromide, 0.1 and 0.2 mg./kg. St, stimulation of central stump of cervical sympathetic nerve for 5 sec. with rectangular pulses of 10 msec. duration at a frequency of 50 c./sec. and intensity of 150 mA. C, hexamethonium bromide, 5 mg./kg. The effect of the ester is blocked by hexamethonium, showing that this is the result of ganglionic stimulation.

Lower record: Effect on respiration, blood pressure and contractions (SG) of the gastrocnemius elicited by supramaximal stimulation of the sciatic nerve. Cat, 2.1 kg.; anesthetic, time, and pressure as above. S_3, senecioylcholine bromide, 0.25 mg./kg.; E, eserine salicylate, 0.15 mg./kg. The ester causes a neuromuscular blockade that is somewhat intensified by eserine, suggesting a depolarizing type of blockade.

Similar methods have led to the identification of the active ester from *B. undatum* as the parent ester of the series, acrylylcholine (IV), although the

$$CH_2{=}CHCO_2CH_2CH_2\overset{+}{N}Me_3$$

IV

presence of some propionylcholine in this species cannot be excluded.[14]

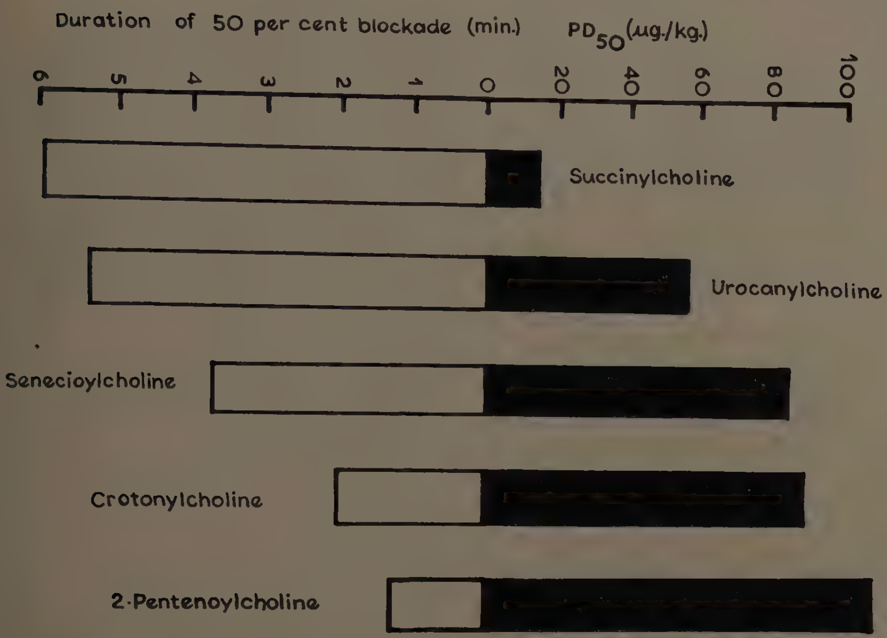


FIGURE 8. Potency of senecioylcholine as a neuromuscular blocking agent compared with succinylcholine, urocanylcholine, and two other β-substituted acrylylcholines. PD_{50}, dose causing 50 per cent blockade when administered by close intra-arterial injection. It will be seen that senecioylcholine is slightly less potent than urocanylcholine, in that the PD_{50} dose is slightly larger and the duration of 50 per cent blockade is shorter.

TABLE 2

PHARMACOLOGICAL EFFECTS OF ACRYLYLCHOLINE BROMIDE

Parameter	Dose (µg./kg.)*	Effect	Relative molar potency (propionylcholine = 1)
Cat			
Muscle blood flow	1	none	0
Heart rate			
normal	1	none	0
atropinized	10–100	increase	—
Blood pressure			
normal	0.1 up	depression	12
eserinized	0.1 up	potentiated depression	1.2
atropinized	10–100	rise	12
Respiration	1–10	stimulation	—
Neuromuscular blockade	130†	46% blockade	5
Guinea pig			
Ileum	10	contraction	5
Frog			
Rectus abdominis muscle	100	contracture	0.2–0.5

* Body weight (cat) or bathing fluid (isolated organs).

† Close arterial injection.

Pharmacological Properties of Snail Esters

Urocanylcholine has a marked hypertensive action and, in man and dog, strongly stimulates the respiration at low doses. It has a neuromuscular blocking action about one fifth that of succinylcholine, and like succinylcholine exerts this effect by a depolarizing action on the end plate region.[8,15,16]

Urocanylcholine has been proposed as an alternative to succinylcholine as a muscular relaxant in clinical use,[17,18] but its nicotinic properties and greater instability would appear to contraindicate this. Senecioylcholine resembles urocanylcholine but is somewhat less active as a neuromuscular blocking agent (FIGURES 7, 8).[19] It is, however, pharmacologically more active than other β-substituted acrylylcholines.

Acrylylcholine resembles propionylcholine in pharmacological properties. It has only an extremely brief and feeble neuromuscular blocking action; it has a greater smooth muscle stimulating and hypotensive action than propionylcholine but, like the latter, is primarily ganglion-stimulating in its action (TABLE 2).*

In keeping with the primitive state of marine invertebrate pharmacology, almost nothing is known of the pharmacological or physiological function of the snail esters in the organisms in which they occur. Dubois[3] and Erspamer[20] inclined to the viewpoint that the hypobranchial gland is a venom organ. However, it seems doubtful that this can be true in the case of *Buccinum undatum*, unless other substances, more toxic than acrylylcholine, are also present. Acetylcholine affects the rate of ciliary beat of the gillplates of *Mytilus*;[21] possibly the hypobranchial secretion plays a similar role in these Rachiglossae.

Senecioylcholine has also been found in the garden tiger moth *Arctia caja*. The choline ester described by Bisset *et al.*[22] has proved to be identical with synthetic senecioylcholine in a number of pharmacological, chromatographic, and spectroscopic tests. The occurrence of the same pharmacologically active substances in two such widely different species poses a fascinating problem in comparative biochemistry.

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PRELIMINARY STUDIES ON THE VENOM OF THE MARINE SNAIL *CONUS**

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One of the most virulent but least studied of animal venoms is that produced by marine snails belonging to the genus *Conus*. This genus contains several hundred species, most of which occur in shallow water in tropical regions. *Conus* is especially abundant on coral reefs throughout the vast Indo-Pacific region.

In so far as is known (Alpers, 1932; Kohn, 1959), all species of *Conus* are predaceous. Hermitte (1946) proposed that the venom is injected into the prey prior to feeding. This was recently confirmed by observation (Kohn 1956, 1959). The prey animal, often a more active species than the snail, is paralyzed by the venom and swallowed whole. Although numerous cases of human injury, and five of fatality, have been reported (Clench and Kondo, 1943; Hermitte, 1946; Kohn, 1958) from *Conus* stings, the venom apparatus is probably not used defensively.

In this paper, we report preliminary studies that have been undertaken in an effort to begin to elucidate the nature of the venom and to understand the mechanism of its use by the mollusk.

Historical aspects. The venomous nature of *Conus* was first recorded by Adams (1848) who described a case of human injury from the sting of *C. aulicus* Linnaeus. Adams concluded correctly that the venom was introduced by the barbed radula tooth of the snail (FIGURE 1). Except for additional reports of human injuries (Clench and Kondo, 1943), the matter does not seem to have been studied at all during the ensuing half century.‡

Morphology of the venom apparatus. The venom apparatus and its component organs were named and described by Bouvier (1887) and Bergh (1895).

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† Experimental work by this author was carried out at the Hawaii Marine Laboratory, University of Hawaii, Honolulu, Hawaii.

‡ It has been recently pointed out to the authors by S. P. Dance of the British Museum (Natural History), London, England, that the venomous nature of *Conus* was known to the great Dutch naturalist, G. E. Rumphius, who reported a human fatality from the sting of the species now known as *C. textile* Linnaeus in his *D'Amboinsche Rariteitkammer* of 1705. This account was recently translated by W. S. S. Van Benthem Jutting (*Rumphius Memorial Volume*, 1959, H. C. D. De Wit, Ed., p. 185, as follows: "It (*C. textile*) is suspected as venomous from the experience in Banda of a female slave. She kept this shell in her hand after she had picked it up when hauling in the seine at sea. As she went to the beach she felt a faint tickling sensation in the hand which crept slowly through her entire body—she died on the spot."

Other accounts have been given by Shaw (1914), Clench and Kondo (1943), Hermitte (1946), and Hinegardner (1958), and the morphology will be but briefly summarized here.

Venom bulb. The most conspicuous organ of the venom apparatus is the venom bulb, a large transversely oriented sausage-shaped organ lying dorsally just in front of the viscera (FIGURES 2 and 3). Some workers (Bergh, 1895; Shaw, 1914) have assumed this to be the source of the venom but Bouvier (1887), Hermitte (1946), and Hinegardner (1958) concluded from histological studies that this was unlikely. The latter view is confirmed by the experiments reported below.

The venom bulb consists mainly of thick layers of muscle, some connective tissue, and a single layer of cuboidal (according to Hermitte) epithelial cells lining a central longitudinal lumen (FIGURE 4). Secretion by the epithelial lining appears to be of minor importance; sections of the venom bulb typically show (FIGURE 4) the lumen to be empty (Hermitte, 1946; Hinegardner, 1958). Bouvier (1887) proposed that the venom bulb functions as a propulsive organ and Hermitte concluded its function to be storage and propulsion of the venom from the site of its production to the radula tooth. This interpretation is probably correct, but means of testing it critically have not been devised as yet.

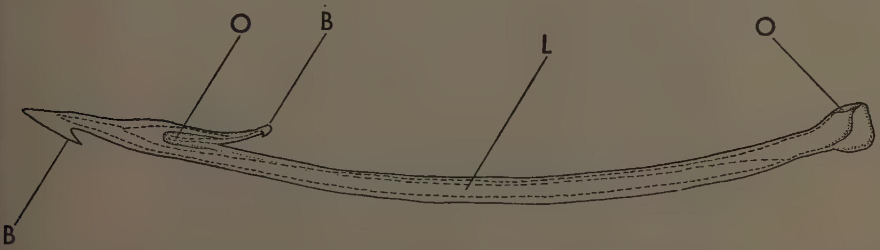

FIGURE 1. Radula tooth of *Conus striatus* Linnaeus, length 10.9 mm. B, barbs; L, lumen; O, openings into lumen.

Venom duct. The venom duct, or tubular poison gland (Hermitte, 1946), is a long, irregularly coiled tube that originates on the right end of the venom bulb and occupies much of the body cavity dorsal and lateral of the esophagus and anterior of the venom bulb. Its distal end enters the anterior portion of the esophagus-stomach, sometimes called the pharynx (FIGURE 3). The length of the duct may be 15 times the straight line distance between its origin and site of insertion (measured in *C. quercinus* Solander), and may exceed 5 times the length of the shell (measured in *C. marmoreus* Linnaeus).

The wall of the venom duct consists of layers of muscular and connective tissue. Within the wall are numerous large, radially-arranged columnar epithelial cells, with basal nuclei and granular cytoplasm (FIGURE 5). Some earlier workers (Bergh, 1895; Shaw, 1914) believed the duct functioned only in transport of the venom to the pharynx. Hermitte (1946) and Hinegardner (1958) concluded on histological grounds that these cells were the source of the venom. The histochemical and toxicological findings reported below support this conclusion. The extreme length of the duct is probably best explained as an adaptation that increases the secretory surface and hence the volume of venom produced.

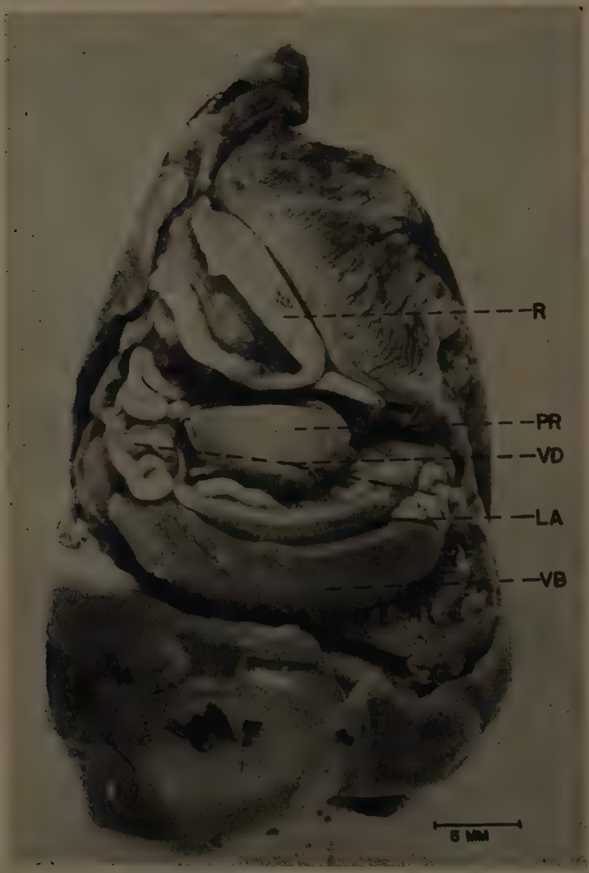

FIGURE 2. Photograph of *Conus textile* Linnaeus, with shell and dorsal body wall removed; LA, long arm of radula sheath; PR, proboscis (retracted); R, proboscis sheath or rostrum; VB, venom bulb; VD, venom duct. Reprinted by permission of the *Hawaii Medical Journa* (Hinegardner, 1958).

Radula. The highly modified radular apparatus of *Conus* is contained in a conspicuous Y- or L-shaped organ, the radula sheath, found dorsally usually just in front of the venom bulb. A longer arm ends blindly; the shorter enters the pharynx on the right side just anterior to the entrance of the venom duct (FIGURE 3).

The long arm contains two rows of about 15 hollow, needlelike teeth oriented with the points directed toward the blind end. The shorter arm contains a smaller bundle of teeth, often about 10, which have their points directed toward the opening into the pharynx. It is likely that the teeth are produced in the long arm, where they are found in various stages of development, and then moved into the shorter arm when fully formed.

In feeding, one tooth (FIGURE 1) is presumably moved from the radula sheath into the pharynx; prior to injection it lies within the distal portion of the proboscis (FIGURE 3). Hermitte proposed a combination of muscular action of the radula sheath and retraction and invagination of the proboscis to grasp the projecting tooth to account for the movements of the tooth, but the mechanism has not been closely studied. While the tooth is in the pharynx or proboscis its lumen is presumably filled with venom, which enters from the

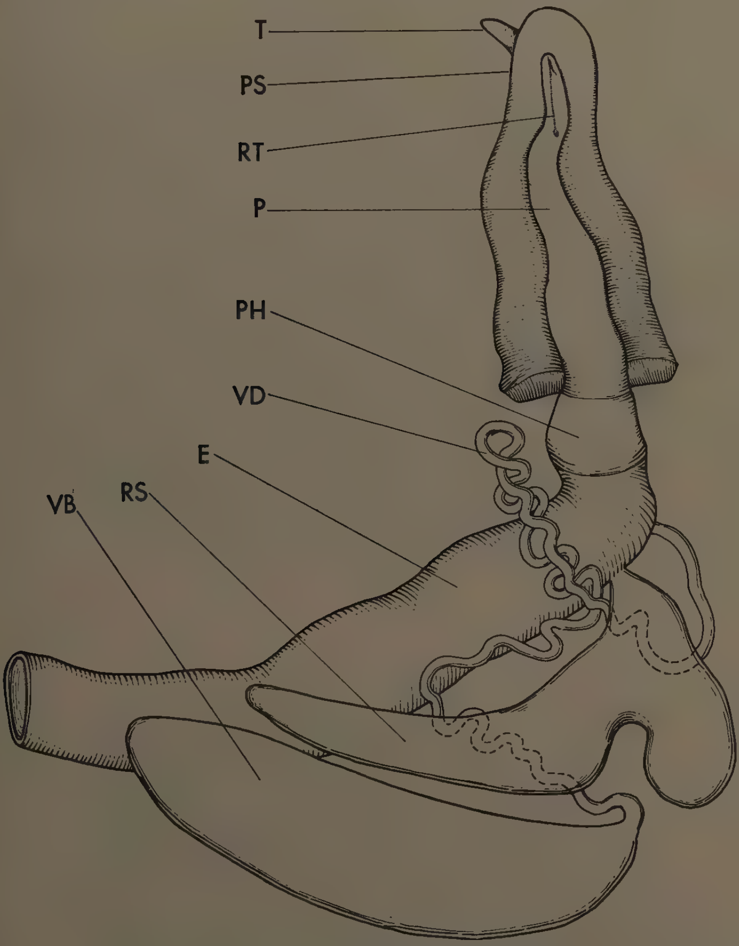

FIGURE 3. Generalized, semidiagrammatic drawing of the organs of the venom apparatus and anterior portion of the digestive tract of *Conus*. E, esophagus-stomach; P, proboscis; PH, pharynx; PS, proboscis sheath; RS, radula sheath; RT, radula tooth within lumen of proboscis; T, tentacle; VB, venom bulb; VD, venom duct.

venom duct. Venom may also adhere to the outside of the tooth. Prior to feeding, the proboscis is extended out of the mouth, its length often approximating that of the shell. The tooth is then injected into the prey which, impaled by the barbs, becomes paralyzed by the venom and is swallowed whole

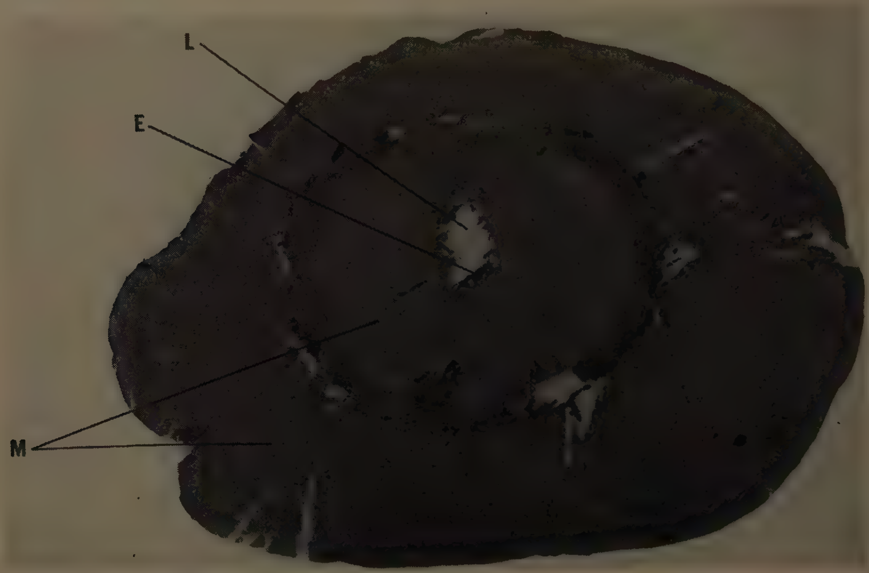

FIGURE 4. Transverse section of venom bulb of *Conus pennaceus* Born, 2.9 × 2.1 mm. Hematoxylin and eosin. E, epithelium; L, lumen; M, muscle.

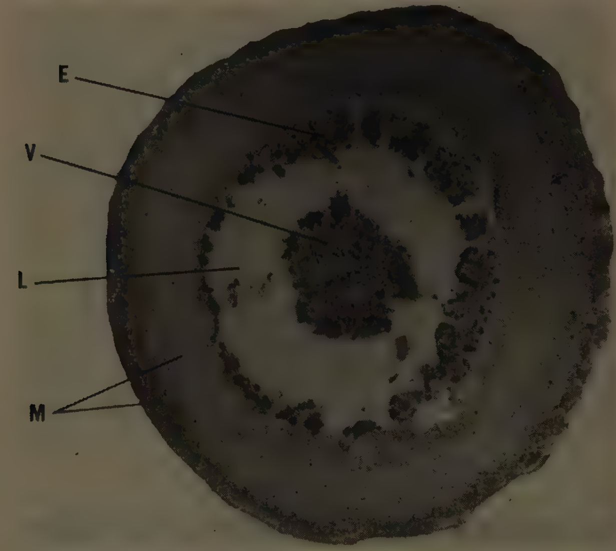

FIGURE 5. Transverse section of venom duct of *Conus striatus* Linnaeus, diameter 0.67 mm. Hematoxylin and eosin. E, epithelium; L, lumen; M, muscle layers; V, venom.

by the predator. The process is reported in more detail elsewhere (Kohn, 1956).

TOXICITY EXPERIMENTS ON MOLLUSKS

Materials and methods. Thirteen specimens of *Conus textile* (mean length, 60 mm.), collected at Guam and shipped via air to California in sea water contained in polyethylene bags, were used in the present work. The specimens were kept alive in an aquarium at 26° C. for periods up to 1½ years, during which time they stung and fed upon a variety of gastropods (*Conus californicus* Hinds, several species of *Nassarius*, and others) found off the coast of southern California. Venom bulbs and ducts, as well as radula sheaths, were removed from living animals and extracts prepared at room temperature by mincing the tissue in sea water, or in sea water diluted with an equal volume of deionized water. The extracts were centrifuged 5 min. at 500 g and bioassayed immediately by injection into the foot of *Conus californicus* Hinds (mean length 19 mm.), *Nassarius tegulus* (Reeve) (mean length 16 mm.), and *N. fossatus* (Gould) (mean length 43 mm.). Injected animals were observed for 48 hours and were scored as dead if no movement occurred for a 24-hour period. The natural food of *C. textile* is other gastropod mollusks, including other species of *Conus* (Kohn, 1959).

TABLE 1

EFFECT OF EXTRACTS OF VENOM APPARATUS OF *CONUS TEXTILE* INJECTED INTO OTHER MARINE SNAILS

Test species	Nature of injection	Volume of extract from specimen (ml.)	Volume of extract injected (mm.^{3})	Total volume of injection (mm.^{3})	No. of snails injected	No. dead in 48 hr.
Conus californicus	duct extracted in sea water (6)*	0.7	20	20	16	16
Conus californicus	duct extracted in 50% sea water (4)	1.0	20	20	12	10
Conus californicus	duct extracted in 50% sea water (4)	1.0	4	20	6	5
Conus californicus	duct extracted in 50% sea water (4)	1.0	0.8	20	3	3
Conus californicus	bulb extracted in sea water (1)	0.6	20	20	6	0
Conus californicus	bulb extracted in 50% sea water (2)	1.0	20	20	3	0
Conus californicus	radula sheath extracted in sea water (5)	0.7	20	20	7	0
Nassarius tegulus	duct extracted in sea water	1.0	20	20	4	3
Nassarius fossatus	duct extracted in sea water	1.0	40	20	2	2

* The number of extracts prepared, each of which utilized one specimen, is shown in parentheses for each extraction medium.

Results. The results are summarized in TABLE 1. As little as about 1/1000 of the total extract obtained from the venom duct of one specimen of *C. textile* was lethal to *C. californicus*. Smaller numbers of *Nassarius* spp. tested were killed. The animals withdrew into their shells immediately after injection and in many cases did not reappear during the observation period. Others extended the foot after several hours but were unable to right themselves and died during the observation period. Specimens of *C. californicus* that had been stung by *C. textile* responded similarly and less than 10 per cent survived.

Animals injected with extracts of the venom bulb and radula sheath, as well as control animals injected with similar volumes of extraction medium, also withdrew into their shells immediately after injection, but always reappeared within 30 min. and then appeared completely recovered during the subsequent 48 hours.

TOXICITY EXPERIMENTS ON CRABS AND FISHES

Material and methods. Specimens of *Conus striatus* Linnaeus collected in Hawaii and Guam were kept in laboratory aquaria without food for 1 to 4 months prior to testing. To obtain known volumes of venom, venom bulbs and ducts were removed from living animals into Stender dishes of sea water and measured lengths of duct cut off under a dissecting microscope. The contents, which were white and granular, were then squeezed out and taken up with sea water in a hypodermic syringe with a No. 27 needle for injection. The lumen of venom bulbs contained so little material that the contents of the entire bulb were squeezed out and taken up in the syringe.

In preliminary experiments, toxicity of the venom of *C. striatus* on the shore crab *Metopograpsus messor* (Forskal) was tested by injection into the leg. In more extensive experiments, venom was injected into the peritoneal cavity or epaxial musculature of the fishes *Neomyxis chaptalii* (Eydoux and Souleyet) and *Mugil cephalus* Linnaeus, both mullet, and the fish *Kuhlia sandvicensis* (Steindachner), or aholehole.

Results with Metopograpsus messor. Three separate experiments were carried out in which 10 of 17 specimens injected with 0.4 to 10 mm.^{3} of *Conus striatus* venom duct contents died within 24 hr. after injection. These results are summarized in TABLE 2. Four of 17 control animals similarly injected with sea water also died within 24 hr. Because of the rather equivocal results, and because of the greater biological significance of experiments with typical prey of *C. striatus*, *M. messor* was abandoned as an assay animal in favor of fishes.

Results with fishes. Intramuscular injection of as little as 0.2 mm.^{3} of the contents of *C. striatus* venom ducts produced a typical syndrome followed by death within 10 min. of injection in two species of fishes (TABLES 3 and 4). *Kuhlia sandvicensis* evinced immediate extension of the dorsal spines and rays, blanching of the entire animal, loss of balance control, and violent convulsions prior to death. The reaction in *Mugil cephalus* was similar, except that convulsions were less pronounced and quivering of the whole body was observed just prior to death. Intraperitoneal injections in *K. sandvicensis* (TABLE 3) and in the mullet *Neomyxis chaptalii* resulted in essentially similar symptoms. Four specimens of *N. chaptalii* injected with about 10 mm.^{3} of venom died within 3 min.

Contents of *C. striatus* venom bulbs injected into *Kuhlia* produced only mild immediate symptoms, but all specimens became moribund about 40 min. after injection. Death occurred 1.5 to 17 hours after injection (TABLE 5), indicating that some toxic material is present in the venom bulb. However only very small amounts of material are found in the lumen of the bulb, and it is not known whether it is secreted *in situ* or enters the bulb from the duct.

TABLE 2

SUMMARY OF THREE EXPERIMENTS ON EFFECT OF VENOM OF *CONUS STRIATUS* ON THE CRAB *METOPOGRAPSUS MESSOR*

Site of injection	Nature of injection	Volume of venom injected (mm.^{3})	Total vol. of injection (mm.^{3})	No. of crabs injected	Effects 24 hr.	Effects 48 hr.
coxa	duct contents	0.43	50	4	normal 1	1
					alive 1	0
					dead 2	3
femur	duct contents	0.57	100	4	normal 0	not observed
					alive 2	
					dead 2	
coxa	duct contents	ca. 10	20	9	normal 2	not observed
					alive 1	
					dead 6	
coxa	bulb contents	undet.	50	2	normal 1	1
					alive 0	
					dead 1	1
femur (2) coxa (14)	sea water	0	20–100	16	normal 11	not observed
					alive 1	
					dead 4	

TABLE 3

EFFECT OF *CONUS STRIATUS* VENOM INJECTED INTO THE FISH *KUHLIA SANDVICENSIS*

Site of injection	Nature of injection	Volume of venom injected (mm.^{3})	Total volume of injection (mm.^{3})	Number of fish injected	Length (mm.)	Effects
epaxial muscles	duct contents	0.6–1.3	20–30	4	46–66	all dead in 3–4 min.
peritoneum	duct contents	10–20	20–40	6	27–74	4 dead in 2.5–10 min. 1 dead in 1 hr. 1 dead in 7 hr.
Controls						
peritoneum	sea water	0	20	4	ca. 40	1 dead, 3 normal after 24 hr.
peritoneum and epaxial muscles	sea water	0	40+50	3		all normal after 24 hr.

Toxicity Experiments on Mice

Series A: Effect of Sea Water Extracts of the Venom of Conus textile

Material and methods. Venom extracts of *C. textile* from Guam were prepared as in the experiments on mollusks described above, and tested by injection into the tail vein of white mice of mean weight of 23 gm. Surviving mice were observed for one week in this and all other series.

Table 4

Effect of *Conus striatus* Venom Injected into the Fish *Mugil cephalus*

Site of injection	Nature of injection	Volume of venom injected (mm.^{3})	Total volume of injection (mm.^{3})	Number of fish injected	Length (mm.)	Effects
epaxial muscles	duct contents	0.2–1.5	20–40	10	49–65	all dead in 5–10 min.
Controls						
epaxial muscles	sea water	0	20–50	10		1 dead in 2 hr., rest normal after 11 hr.

Table 5

Effect of *Conus striatus* Venom Bulb Contents Injected into *Kuhlia sandvicensis*

Number of fish	Site of injection	Volume of venom injected (mm.^{3})	Total volume of injection (mm.^{3})	Effects
3 (54–61 mm.)	epaxial muscles	—	30–50	all moribund in 40 min. 2 dead in 5 hr., 3rd dead in 17 hr.
3 (25–28 mm.)	peritoneum	—	20	all dead in 1–5 hr.

Table 6

Effect of *Conus textile* Venom Apparatus Extracts Injected into Mice

Nature of injection	Volume of extract from specimen (ml.)	Volume of extract injected (mm.^{3})	Total volume of injection (mm.^{3})	Number of mice injected	Number dead in 48 hr.
duct extracted in sea water	0.7	80–100	80–100	4	0
duct extracted in 50% sea water	1.0	50–300	50–300	10	2*
bulb extracted in 50% sea water	1.0	250	250	1	0
radula sheath	0.7	200	200	2	0

* The two specimens that died received injections of 200 mm.^{3} of extract.

Results. Two deaths occurred in mice following injection of 0.2 ml. of venom duct extract (TABLE 6). No death occurred following injection of venom bulb and radula sheath extracts, although about half of the mice displayed mild effects (inactivity, eyes watering) for about 30 min. The two mice that died approximately 45 min. after injection showed similar symptoms initially, followed by marked inactivity, apparent paralysis of limbs, and loss of righting reflex immediately prior to death.

Control mice injected with volumes of extraction medium equal to those used in the venom tests showed no effects.

Series B: Effect of Normal Saline Solution Extracts of Venom of Conus textile

Material and methods. Contents of venom ducts and bulbs of specimens collected at Heron Island, off Queensland, Australia, were extracted in normal saline solution and the supernatant thus obtained was injected into mice by the intraperitoneal and intravenous (tail vein) routes. All test animals in Series B through E were white mice, weighing 15 to 20 gm. They were observed for one week following injection.

Results. No toxic effects were observed when supernatants obtained from venom ducts of two *C. textile* were injected into two mice, one intraperitoneally and the other intravenously. However, intraperitoneal injection of supernatant from a third specimen produced the following effects: flushing of tail and paws, sluggish movements, and weakness of hind legs, followed by paralysis and coma. Death occurred 2½ hours following the injection. At post-mortem there was fluid in the peritoneal cavity, the lungs were normal, and there was fluid blood in the heart. Some of the peritoneal fluid was aspirated and injected into another mouse; it produced no toxic effects.

One mouse was injected intravenously with supernatant from a second extraction of the precipitate in normal saline solution. About one hour later the animal had slight weakness of the hind limbs, from which it completely recovered during the next three hours. One mouse was injected intraperitoneally with supernatant from a third extract of the precipitate in normal saline solution. Twenty minutes later the animal had muscular weakness and ataxia. Loss of righting reflex and failure to react to painful stimuli occurred four hours later. The eyes remained open and the heart continued to beat vigorously, but breathing consisted of shallow gasps. Coma then supervened and the animal died four hours after injection. Apart from slight congestion of the intestine, no abnormalities were observed at post-mortem. The results of these and subsequent experiments in this series are summarized in TABLE 7.

Series C: Effect of the Venom of Conus striatus Linnaeus

Material and methods. Living specimens of *C. striatus* from Queensland were frozen and stored at −15° C. Venom was obtained by extracting the venom duct of thawed animals in normal saline solution or distilled water. In the former case the extract was injected immediately into mice by the subcutaneous and intracerebal routes. Distilled water extracts were freeze-dried and redissolved in normal saline solution before use. The small amount of material present in the lumen of the venom bulb was also injected.

The contents of the venom duct of one specimen were expressed into 0.5 ml. of normal saline solution and the resulting suspension was injected into a mouse by the subcutaneous route. Within 10 min. the animal appeared sick and then developed the following signs: ataxia, tonic spasms, dyspnea, hyperexcitability, and paresis. Death occurred 68 min. after the injections, from failure of respiration followed by cardiac arrest. On post-mortem examination the lungs were hemorrhagic in appearance and the heart contained fluid blood that did not clot for a period of 10 min. after some of it had been transferred into a glass tube. There was no hemolysis.

Material present in the venom bulb was injected subcutaneously into one mouse, which died 47 min. later. In addition to the symptoms that resulted from injection of venom duct contents, this animal also failed to react to painful stimuli, although the blinking reflex remained intact.

The ducts of four female and two male specimens (shell length about 3 inches; length of venom duct 5 to 6 inches) were extracted in distilled water and freeze-dried. It was found necessary to repeat the extraction process at least 5 times in order to dissolve all the toxic material present in the duct. The amount of dried venom obtained ranged from 2.6 mg. to 10.5 mg., with an average of 6.7 mg. of venom per animal.

TABLE 7
EFFECT OF *CONUS TEXTILE* VENOM DUCT EXTRACTS IN NORMAL SALINE SOLUTION INJECTED INTO MICE

Mode of injection	Nature of injection	Number of mice injected	Reaction
intravenous	supernatant	1	none
intraperitoneal	supernatant	1	none
intraperitoneal	supernatant	1	death in 2½ hr.
intravenous	supernatant of 2nd extraction	1	weakness, followed by recovery in 3 hr.
intraperitoneal	supernatant of 3rd extraction	1	death in 4 hr.
various routes	whole extract	8	none in 6; one dead in 24 hr. (intravenous); weakness followed by recovery in one (intraperitoneal)

Typical results of intracerebral injection of freeze-dried *C. striatus* venom are shown in TABLE 8. The LD$_{50}$ ranged from 0.5 to 1.0×10^{-4} mg.

After an intracerebral injection of lethal amounts of venom, the animals were hyperexcitable and jumped or raced around in their cages. Dyspnea was evident, and frothy fluid, often blood-stained, discharged from their noses. Tremors, ataxia with marked flexion of the head, and violent scratching movements were frequently present. Death was preceded by convulsions and occurred either immediately or within three hours, depending on the dose of venom injected.

Dyspnea was particularly noticeable in the animals that lived longer than 30 min. after the injection of lethal amounts of venom. In some of these cases the animals assumed a peculiar posture. They sat on their haunches, supported by the front legs, while the hind legs were extended in front. With each inspiration the head moved upwards through an arc of one or two inches, and then came down again during expiration.

With sublethal doses of venom some of the animals had ataxia with a stilted gait and dyspnea; they recovered during the next 24 hours.

On post-mortem examination only the lungs showed abnormalities. They were either hemorrhagic and resembled a clot of blood or emphysematous and pale in color without hemorrhage. These differences in the appearance of the lungs could not be related to the amount of venom injected nor to the time that had elapsed between injection of venom and death.

Toxic effects were also observed when reconstituted freeze-dried venom was injected by the intraperitoneal and subcutaneous routes. The animals showed no excitatory phenomena but several or all of the following signs were present when lethal amounts of venom were injected: flaccid paralysis, tonic spasms, dyspnea, ataxia, hypothermia, and coma. Death was usually heralded by progressive failure of respiration without the occurrence of convulsions.

Because of lack of sufficient venom only a few quantitative studies were carried out. The lethal dose of venom by the intraperitoneal and intravenous routes ranged from 0.1 to 0.3 mg. of venom per mouse. However, the freeze-dried venom of one specimen was not toxic when up to 0.6 mg. was injected by the intraperiteoneal route, although by the intracerebral route the lethal dose was 0.5×10^{-4} mg. Following the subcutaneous injection of 0.5 mg. of venom, a mouse died in 2 hours.

TABLE 8

TOXICITY OF THE VENOM OF *C. STRIATUS* INJECTED INTO MICE BY THE INTRACEREBRAL ROUTE

Amount of venom in mg. $\times 10^{-4}$	Number of mice injected	Number of mice that died
32	4	4
16	4	4
8	5	5
4	5	5
2	5	5
1	5	4
0.5	5	2
0.25	5	0
0.12	4	1
0.06	4	0

Series D: Effect of the Venom of Conus aulicus Linnaeus

Material and methods. Venom ducts of specimens collected in Queensland were extracted in normal saline solution or distilled water and centrifuged. The latter extracts were freeze-dried, then redissolved in normal saline solution prior to injection.

Results. One animal injected subcutaneously with 1.9 ml. of venom duct extract in normal saline solution was reluctant to move two hours after injection; this was followed by flaccid paralysis of the hind limbs and the presence of tears in the eyes. Next morning the animal was found dead in its cage. No distinctive post-mortem changes were observed. Five mice injected with lesser amounts via other routes evinced no symptoms or only minor ones, followed by recovery. One individual injected intravenously with the contents of the venom bulb suffered no ill effects.

Series E: Effect of the Venom of Conus marmoreus

Material and methods. Nine specimens from Queensland were treated as in the previous series.

Results. Intraperitoneal injection of 0.4 ml. of supernatant from normal saline solution extracts produced no immediate toxic effects. However the one mouse so injected was found dead the following morning and both its lungs were hemorrhagic in appearance. The rest of the supernatant was stored at 4° C. overnight, after which 0.6 ml. and 0.25 ml. were injected into two mice by the cutaneous and intravenous routes respectively. Both animals remained well. Intracerebral injection of 0.01 ml. of the same extract resulted in ataxia, paralysis, and death of all three mice so tested. Intravenous injection of the whole supernatant from one venom duct extract into a mouse produced no toxic effects.

Mice tolerated up to 25 mg., 3 mg., and 0.15 mg. of redissolved freeze-dried venom injected by the intraperitoneal, intravenous, and intracerebral routes, respectively.

No toxic effects were observed when the contents of three venom bulbs were injected into mice by the intraperitoneal and subcutaneous routes.

Other Experiments

Extracts of the venom duct and venom bulb of one specimen of each of the following species were made in the manner described above and injected into mice by the intraperitoneal and intravenous routes: *C. flavidus* Lamarck, *C. leopardus* (Röding), and *C. maldivus* Hwass *in* Bruguière, from Queensland. No toxic effects were observed from any of these extracts.

Intraperitoneal injection of 30 mg. of redissolved freeze-dried *C. marmoreus* venom into a guinea pig weighing 250 gm. produced no toxic effects. Intradermal injection of 0.2 mg. of the same extract into another guinea pig produced only a slight reddening at the site of injection. Intraperitoneal injection of 0.6 mg. of redissoved freeze-dried *C. striatus* venom into a guinea pig (weight 250 gm.) produced no toxic effects.

No differences in the toxicity of the venom of male and female *C. striatus* were observed.

Venom of *C. striatus* did not cause hemolysis when 1 mg. was added to 0.5 ml. of a 3 per cent suspension of mouse erythrocytes.

CHARACTERISTICS OF THE VENOM

Appearance. Material expressed from the venom duct of all species examined is usually white or pale yellow, but occasionally appears gray or black in *C. striatus*. The consistency is viscous, and the material appears granular under microscopic examination. In *C. striatus* some granules are almost circular, measuring 2 to 3 μ in diameter; others, measuring 3 × 7 μ, appear oval. The granules stained yellow with iodine were insoluble in alcohol and resisted boiling with concentrated HCl, but dissolved in an aqueous solution of

20 per cent KOH. The freeze-dried venom preparations used in some of the experiments reported above did not contain these bodies, since they were discarded with the precipitate after the final extraction.

Freshly collected venom of *C. striatus* had a *p*H of 8.

Stability of extracts stored in frozen state. Extracts of venom ducts of *C. textile* in 50 per cent sea water were stored at −20° C. for three weeks. Subsequent injection into *C. californicus* produced effects apparently identical with those obtained with fresh extracts.

Heat stability. Venom from ducts of *Conus striatus*, heated 10 min. at 90 to 100° C., changed color from white to dark yellow and appeared to coagulate. Nine of 13 *Kuhlia sandvicensis* injected with heated venom extract (cooled to 25° C.) died after 2 min. to 7 hours (TABLE 9). Those injected intraperitoneally evinced rapid swimming movements or convulsions; intramuscular injections produced noticeably increased respiratory rate and color changes in which the dorsum showed yellow and dark gray blotches, with a dark gray area around the site of injection. Individuals injected intramuscularly with heated (and cooled) bulb extract showed similar but less pronounced color changes and respiratory effects and appeared to recover completely. Heating thus appears to alter but not destroy the toxicity of the venom to fishes. *C. striatus* venom heated 10 min. at 100° C. did not lose its toxicity to mice when injected by the intracerebral and intraperitoneal routes.

TABLE 9

EFFECT OF HEATING (10 MIN. AT 90–100° C.) ON TOXICITY OF *CONUS STRIATUS* VENOM

Assay animal	Number	Site of injection	Nature of injection	Volume of venom injected (mm.^{3})	Total volume of injection (mm.^{3})	Effects
Kuhlia sandvicensis (27–34 mm.)	8	peritoneum	duct contents	ca. 10	20	2 dead in 2–3 min. 4 dead in 2–5 hr. 2 normal after 24 hr.
Kuhlia sandvicensis (45–59 mm.)	5	epaxial muscles	duct contents	0.9	30–70	Immediate color change, increased resp. rate, followed by: 2 smallest dead in 2 hr., 1 dead in 7 hr., 2 normal after 24 hr.
Kuhlia sandvicensis	5	epaxial muscles	bulb contents	—	40–60	Immediate color change, increased resp. rate, followed by complete recovery after 4 hr. All normal after 21 hr.

Extracts of venom ducts of *C. textile* in 50 per cent sea water were heated for 5 min. in a boiling water bath, during which period a white precipitate formed; the supernatant fraction obtained after centrifugation was still lethal to *C. californicus.*

Effect of dialysis. About 5 mm.^{3} of *Conus striatus* venom duct extract was dialyzed against distilled water at 10° C. for 19 hours. Intramuscular injection of the contents of the dialysis tubing (warmed to 25° C.) into 6 fishes (3 *K. sandvicensis*, 3 *M. cephalus*) produced symptoms indistinguishable from those obtained with untreated venom (TABLES 3 and 4). All of the fishes died 4 to 8 min. after injection. The active substance(s) is (are) therefore nondialyzable, suggesting a possible role of protein.

Effect of peptidase. *C. striatus* venom extracts prepared as above were incubated with approximately equal volumes of trypsin in sea water (*p*H 8.1 to 8.3) for 3 to 6 hr. at 25° C., and injected into fishes. In one experiment with *Kuhlia* (TABLE 10), the potency was identical with that of untreated venom (TABLE 3). In *Mugil* (TABLE 10), no symptoms appeared for one to several minutes, following which the fish appeared to lack full voluntary control of movements. Distress was apparent after an average of 11 min., but death did not follow until an average of 18 min. after onset of distress. The latter experiments also suggest a partial role of protein in the active principle of *Conus* venom. *C. striatus* venom extracts also gave positive biuret, ninhydrin, and xanthoproteic tests.

TABLE 10

EFFECT OF INCUBATION WITH TRYPSIN ON TOXICITY OF *CONUS STRIATUS* VENOM

Assay animal	Number	Nature of injection	Volume of venom injected (mm.^{3})	Total volume of injection* (mm.^{3})	Effect
Kuhlia sandvicensis (38–84 mm.)	5	venom + trypsin† (3 hr.)	0.7	30–50	all dead in 1–10 min.
Mugil cephalus (48–62 mm.)	6	venom + trypsin‡ (3 hr.)	1.5	20	distress in 3–44 min. all dead in 15–64 (mean 28) min.
Mugil cephalus (51–59 mm.)	6	venom + trypsin‡ (6 hr.)	1.5	30	distress in 7–14 min. all dead in 10–55 (mean 32) min.
Controls					
Kuhlia sandvicensis	3	trypsin† in sea water	0	50	all normal after 15 min.
Mugil cephalus	6	trypsin‡ in sea water	0	20	all normal after 7 hours

* All injections were made into epaxial muscles.
† Crystallized trypsin (Armour).
‡ Powdered trypsin (Pfanstiehl).

Presence of carbohydrate. Freshly collected *C. striatus* venom gave a positive Molisch reaction. After acid hydrolysis the Molisch reaction became intensified, but no reducing sugars were detectable.

Histochemistry. Venom ducts and bulbs of *C. striatus* and *C. marmoreus* were fixed in Orth's fixative, sectioned, and examined for the chromaffin reaction. Brownish-yellow to brown granules 8 to 10 μ in diameter were observed within the epithelial cells of the duct of *C. striatus*, (FIGURE 6), but not in *C. marmoreus*. They were also absent from the bulbs of both species. The positive reaction in *C. striatus* suggests the possible presence of toxic amines in the venom and raises the possibility of interspecific differences in the nature of the active substance(s) of *Conus* venom.

Chromatography. Chromatograms of acetone extracts of venom ducts and bulbs of five species of *Conus* were prepared by J. H. Welsh, following the methods described in detail by Welsh and Prock (1958). The solvent consisted of 95 parts of 95 per cent ethanol and five parts of 28 per cent NH_4OH. Runs lasted 6½ to 10 hr., and the ascending method was used with wide strips of Whatman No. 3MM filter paper. After drying, a spray of Dragendorff's solution ($KBiI_4$ reagent) was used to visualize areas occupied by quaternary ammonium compounds.

FIGURE 7 shows a composite of chromatograms prepared from equal amounts of duct and bulb homogenates and from the known quaternary ammonium compounds *N*-methylpyridinium (R_f = 0.64), homarine (R_f = 0.57), and gamma-butyrobetaine (R_f = 0.37).

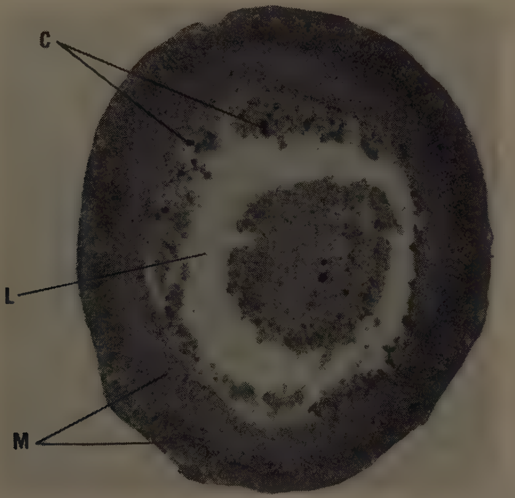

FIGURE 6. Transverse section of venom duct of *Conus striatus* Linnaeus, the same specimen as in FIGURE 5. Average diameter 0.55 mm. Orth's fixative plus hematoxylin and eosin. C, chromaffin-positive granules; L, lumen; M, muscle layers.

It is of interest to note that the bases mentioned above are present in higher concentrations in *C. striatus* and *C. textile* than in *C. litteratus*, *C. marmoreus*, and *C. magus*, which showed pale homarine spots and absence of gamma-butyrobetaine in the latter two. The first two species are known to have relatively very large and well-developed venom apparatuses. Two cases of human fatality from the sting of *C. textile* are known (Clench and Kondo, 1943).

Other chromatograms sprayed with Ehrlich's reagent suggested the presence of indole derivates, which were not identified. J. H. Welsh (in personal communication) has suggested that they are possibly indole amines, which may be responsible for the positive chromaffin reaction mentioned above.

Serology. C. striatus venom in solution was partially adsorbed on aluminum phosphate in suspension at *p*H 5. A rabbit received five injections of Al_3PO_4

with the adsorbed venom increasing from 0.3 mg. to 2.0 mg. at weekly intervals. The rabbit suffered no ill effects. Serum from blood collected from the rabbit one week after the final injection gave a positive precipitin reaction when mixed with venom from *C. striatus* and *C. marmoreus*. The serum also agglutinated a suspension of the granular bodies described above as present in the venom of *C. striatus*. No tests have been carried out to determine if the serum neutralized the toxic effect of the venom.

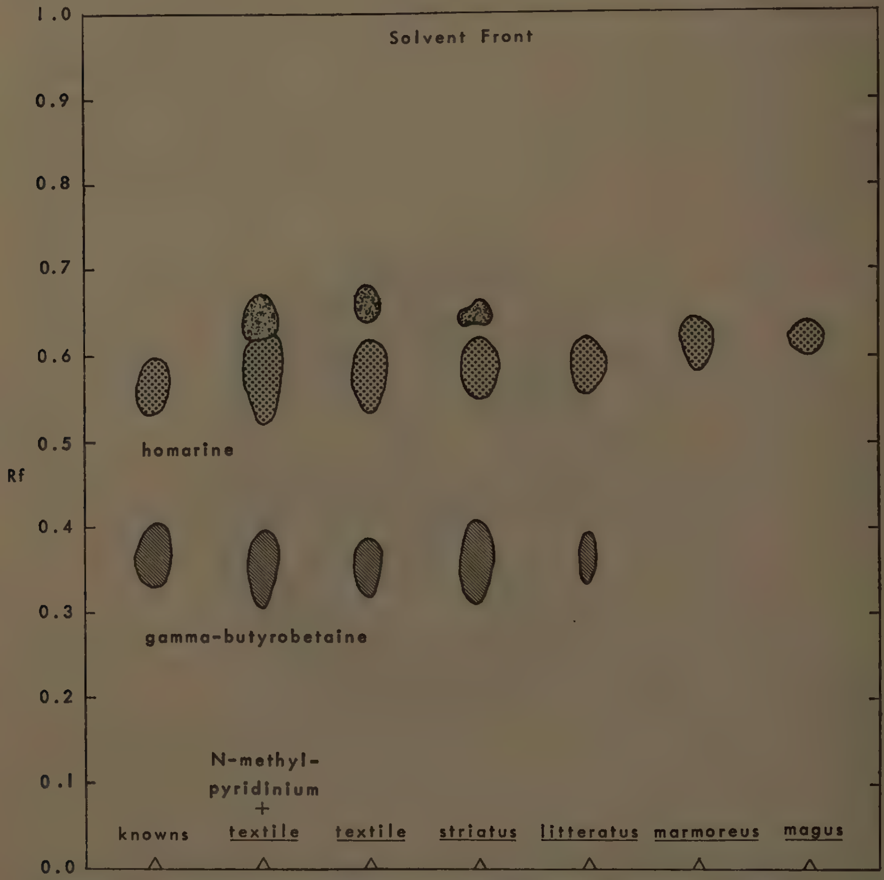

FIGURE 7. Composite of chromatograms showing R_f values of known quaternary ammonium compounds and extracts of venom bulbs and ducts of five species of *Conus*. *N*-methylpyridinium was added to *C. textile* extract, as it is not stable in basic solvent. The mixed chromatogram gave a well defined single spot. The homarine spots of *C. litteratus*, *C. marmoreus*, and *C. magus* were paler than those of the other species.

SUMMARY AND CONCLUSIONS

Injections of small quantities of extracts of the venom duct (tubular venom gland) of several species of *Conus* proved lethal to other mollusks, fishes, and mice. The venom of *C. striatus* was employed in most of the experiments, but toxic reactions were also produced by venom of *C. textile*, *C. aulicus*, and *C. marmoreus*.

The minimum lethal dose of *C. textile* venom, tested on *C. californicus*, is about 1/1000 of the total venom from one specimen, or <0.8 mm.^{3} of extract of whole venom duct. The minimum lethal dose of *C. striatus* venom is <0.2 mm.^{3} of venom duct contents injected into fishes 50 to 65 mm. in length. The LD_{50} of *C. striatus* venom injected into mice is 0.5 to 1.0×10^{-4} mg. of venom duct contents, tested intracerebrally, and 0.1 to 0.3 mg., tested intravenously and intraperitoneally. The minimum lethal dose of whole venom duct extracts of *C. textile*, injected intravenously, is less than 200 mm.^{3} in mice. Fishes usually died less than 10 min. after injection; death in mice occurred within 4 hours after injection.

Extracts of venom bulbs and radula sheaths of *C. textile* and *C. aulicus* did not produce toxic effects when injected into snails and mice. Contents of *C. striatus* venom bulbs were lethal when injected into one mouse and fishes, although death in the latter occurred after one to several hours, in contrast to a few minutes following injection of duct contents.

Histological studies made by us and by previous workers (Bouvier, 1887; Hermitte, 1946; Hinegardner, 1958) suggest that venom is not secreted in the bulb, which is largely muscular and has a narrow central lumen lined by a thin epithelium. It is possible that the bulb functions in pumping venom from the duct into the pharynx. The toxic material found in the lumen in *C. striatus* probably was produced in the duct rather than *in situ*.

Symptoms preceding death of fishes from injection of *C. striatus* venom typically included color change, ataxia, convulsions or quivering, and paresis. Mice injected with *C. striatus* venom developed ataxia, tonic spasms, dyspnea, hyperexcitability, violent scratching movements, and paresis prior to death. Hemorrhage or emphysema of the lungs were the only post-mortem abnormalities observed.

Venom of *C. textile* induced flushing of tail and paws, sluggishness, dyspnea, paralysis, and coma prior to death in mice. Post-mortem abnormalities were minor. Tears, ataxia, and paresis, followed by recovery, occurred in mice injected with venom from *C. aulicus*.

In some experiments, for example in Series C, death of mice could be attributed to failure of respiration followed by cardiac arrest. In others the exact cause of death is unknown, as in the cases of human fatality reported (Kohn, 1958). However, the responses are in general similar to those from curariform drugs (Craig, 1948).

The toxic manifestations suggest the main action of the venom is interference with neuromuscular transmission, although the possibility of an action on the central nervous system cannot be excluded.

In the single experiment performed, *C. striatus* venom had no hemolytic action on mouse erythrocytes.

The crude venom is white or yellow, viscous, and granular in appearance, and has a *p*H of 8. Protein and carbohydrate are present. No activity was lost on dialysis. Heating and incubation with trypsin altered but did not destroy the toxicity; death was usually delayed in fishes injected with venom so treated. Chromatography showed the presence of the quaternary ammonium compounds *N*-methylpyridinium, homarine, and gamma-butyrobetaine, and unidentified indole derivative homogenates in the venom duct. Venom of one species gave a positive chromaffin reaction, suggesting the presence of amines. 5-Hydroxytryptamine was not detected, but other indole amines are possibly present (J. H. Welsh, personal communication).

It is possible that the quaternary ammonium compounds may compete for acetylcholine receptors at the motor-end plate, either preventing acetylcholine from exerting its depolarizing action, as *d*-tubocurarine, or, as decamethonium, producing continuous depolarization, thus preventing acetylcholine, which is rapidly hydrolyzed by cholinesterase, from acting as a physiological depolarizing agent.

N-methylpyridinium is known to possess curariform activity (Craig, 1948). Although injection of *N*-methylpyridinium, homarine, and gamma-butyrobetaine separately into *Uca pugilator* did not induce paralysis (Welsh and Prock, 1958), these authors postulated possible toxicity of a mixture of bases. On the other hand, these compounds are of rather general occurrence in mollusks (J. H. Welsh, personal communication), and may not be concerned with the toxicity of the venom.

The results of toxicity experiments with venom that had been heated, dialyzed, and incubated with proteolytic enzymes suggests a partial role of protein or polypeptide. The absence of hemolytic activity and the symptoms induced in fishes following intramuscular injection are properties similar to those of the nematocyst venom of *Physalia*, which Lane and Dodge (1958) concluded to be a highly labile protein complex. On the other hand the protein present in *Conus* venom may be nontoxic, and the retention of considerable toxicity following heating and incubation with trypsin suggests the presence of other toxic agents.

The preliminary studies suggest the possibility that the venom of *Conus* may contain several active principles, including protein, quaternary ammonium compounds, and possibly amines. This interpretation is similar to that of Welsh and Prock (1958), who state, with reference to cnidarian toxins, "It is not unreasonable to theorize that an association of tetramine with a protein might produce a substance more toxic than tetramine alone. This is based partly on the evidence that two alkylated tetracovalent nitrogens, properly spaced in a molecule, can produce highly active junctional blocking agents such as curare and the many synthetic, curariform, bis-quaternary substances. Protein denaturation by heat, or otherwise, might alter the spacing of the tetramines on the protein or set them free and thereby reduce, but not abolish, the paralyzing action of an extract." However, the suggestions regarding the components responsible for toxicity of the venom of *Conus*, as well as its mode of action, must be regarded as conjectural.

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TOXICITY OF OCTOPUS SALIVA AGAINST CRUSTACEA*

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Introduction

Salvatore Lo Bianco, working at the Naples Zoological Station, in 1888 first indicated that there is a substance in the posterior salivary glands (p.s.gl.) of octopus that is toxic for crabs. Lo Bianco, who was a self-made zoologist, in his *Biological Information on the Time of Sexual Maturity of Animals in the Bay of Naples*[1] gave the following description of how the mollusc attacks his prey:

"Years ago I realised that the Octopus neither kills the crab by the bite of its chitinous jaws nor by means of its tentacles and suckers. It paralyses the prey with a powerful poison secreted by the p.s.gl. As soon as the Octopus perceives the crab, with a rapid movement it covers it with its body and tentacles in such a position as to bring its mouth very close to the branchial hole of the crab, into which it injects the poison. Crabs forcibly released from the Octopus show difficulty in locomotion and violent irregular shaking of the pincers and the thoracic legs, followed a few minutes later by immobility and death."

"I have reproduced these effects experimentally," says Lo Bianco, "by injecting some saliva into the gills of the crab. Never was I able to avoid paralysis and death, even when the animal was washed under running sea water."

These observations were the beginning of a number of morphological, physiological, biochemical, and pharmacological studies by several investigators, including B. Rawitz, R. Krause, M. Henze, C. Livon, L. Joubin, A. Briot, S. Bagioni, F. Bottazzi, E. Sereni and, more recently, Z. M. Bacq, W. G. Clark, M. Vialli, and V. Erspamer. Certainly, the p.s.gl. of Cephalopoda still provide fascinating material for anyone interested in comparative physiology and biochemistry.

In 1906 two French authors, Livon and Briot,[2] working chiefly with *Eledone*, claimed that the poison from the p.s.gl. is a substance of complex nature, probably a kind of albuminoid, as proteins were called at that time. The experimental evidence for this was that the substance does not pass through a dialyzing membrane, and that its activity is destroyed by heating. According to the same authors, the poison is specifically active against the central nervous system of Crustacea. Curiously enough, these observations did not receive any attention at that time, and the work of Livon and Briot was entirely forgotten. In 1913 Henze,[3] working at the Naples Zoological Station, succeeded in isolating tyramine from the p.s.gl. of *Octopus vulgaris* and claimed immediately that this was the crab-paralyzing agent present in the glands and saliva. A few years later Bottazzi,[4,5] working at the same laboratory, suggested that another factor, histamine, might be present, and is responsible, either alone or associated with tyramine, for the paralyzing action against Crustacea. Since

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then a number of substances have been found in the p.s.gl. of Cephalopods; in addition to tyramine, histamine, acetylcholine, and taurine, two new amines were recently discovered in the glands by Erspamer:[6,7,8] *p*-hydroxyphenyl-ethanol-amine (octopamine); and 5-hydroxytryptamine (5-HT, serotonin, enteramine). All these substances are readily diffusible and quite resistant to heating. All have typical pharmacological properties and, at high doses, each of them is toxic for crabs. While every one of these amines, alone or associated with the others, has from time to time been identified with the poison, it is only tyramine that has really been favored in textbooks. According to the textbooks tyramine is the crab-paralyzing agent in the p.s.gl. of Cephalopoda.[9]

However it can be easily demonstrated that neither tyramine nor any other amine present in the glands, when injected into a crab in an amount equal to that present in a lethal quantity of saliva, is able to kill the animal.[10] In addition, different species of Cephalopoda have different contents of phenolic and indolic amines in their saliva,[6,7] even though each is equally toxic. *Eledone moschata* has very little tyramine; *Octopus macropus* has no 5-HT; and *Sepia officinalis* contains neither 5-HT, tyramine, nor octopamine.[11]

Experimental

Collection of material. Coming now to my experiments, I shall mention first the method employed for collecting saliva.[12,13]

Cephalopodal Octopods have two pairs of salivary glands differing in position, size, structure, and chemical composition. The anterior pair is closely attached to the external surface of the buccal mass, whereas the posterior one is situated at the side of the crop (FIGURE 1).

The posterior glands are much larger than the anterior glands, weighing 0.3 per cent of the body weight in *Octopus vulgaris* and in *Eledone moschata.* In *O. macropus* they are proportionately much larger, and investigators have noticed a characteristic sexual dimorphism: the glands are twice as large in males as in females. A single duct leaves the anterior internal face of each gland and, after a short course, joins its fellow to form a common duct that runs forward to the buccal mass. The glands consist of secretory tubules embedded in a stroma of connective tissue. The arterial blood is provided by two arteries while the venous blood passes out directly into the perivisceral venous sinus. The posterior salivary glands are entirely enclosed in a division of this sinus and are washed in the blood contained by it.[14]

The principal steps in the technique used for collecting saliva are isolation by dissection, perfusion with sea water, and electric stimulation of the glands. The method for killing the animal is to bleed it by putting a cannula into the aorta, in the same way as into the carotid artery of a rabbit. Once back in the sea water, the blood comes out at every respiratory movement and can be almost completely drained from the body.

The mantle is cut dorsally and the venous sinus is opened to set free the glands (FIGURE 2). All the small arteries in the region of the esophagus are ligated and cut. The connective ligaments binding each gland to the crop are also tied off and cut. Dissection is carefully continued to the anterior end of the visceral envelope, where the aorta forks. Each branch supplies salivary arteries that run back to the glands. Each vessel is tied very close to its origin. Since the esophagus and common duct actually pass through the brain, it is necessary to cut the supraesophageal part to set them free and they, too, are bound and cut. The organs are thus completely isolated from the body with their vessels and nerves. A small platinum cannula is introduced at the end of the salivary duct and a glass cannula is fixed into the aorta. The glands are placed on a plastic network and connected through the glass cannula to a Mariotte bottle filled with filtered sea water at constant pressure (FIGURE 3). The platinum cannula is joined to wire electrodes. When carefully undertaken, this preparation functions for several hours; it gives a constant flow rate of 3 to 9 ml. of sea water per minute per gram of tissue, and is very sensitive to changes of pressure. The flow can be recorded by means of a drop recording assembly.[15] Rhythmical variations at intervals of 10 to 45 sec. occur due to the rhythmic movements of the vessels and of the smooth muscle of the organs.[16] The flow rate is greatly modified by electrical or mechanical stimulation, as well as by

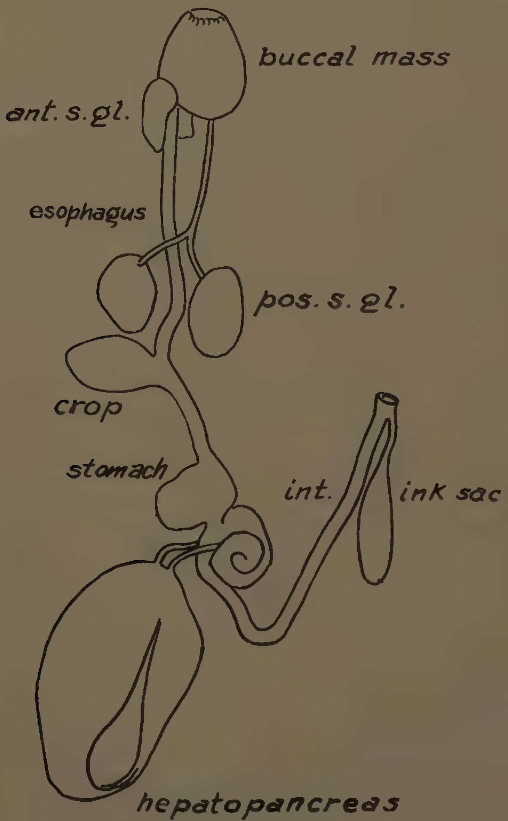

FIGURE 1. *Eledone cirrosa.* Alimentary canal dissected out of the body. From Isgrove, 1909.[14]

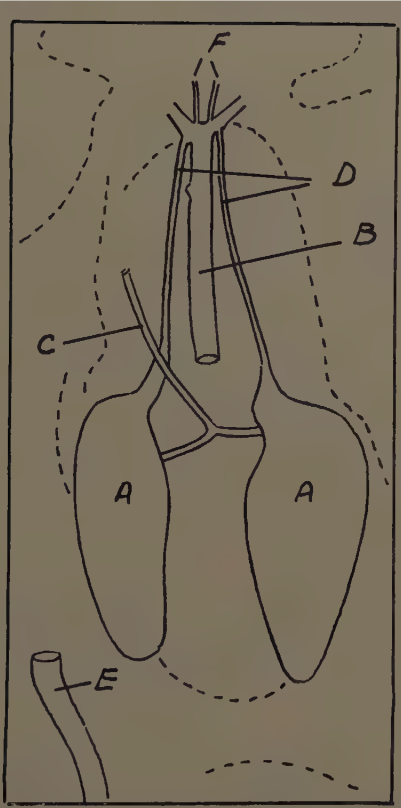

FIGURE 2. Isolation of the posterior salivary glands of *Octopus macropus*. *A* are the posterior salivary glands; *B* is the aorta; *C* is the common duct; *D* are the salivary arteries; *E* is the esophagus; and *F* are the pharyngeal arteries.

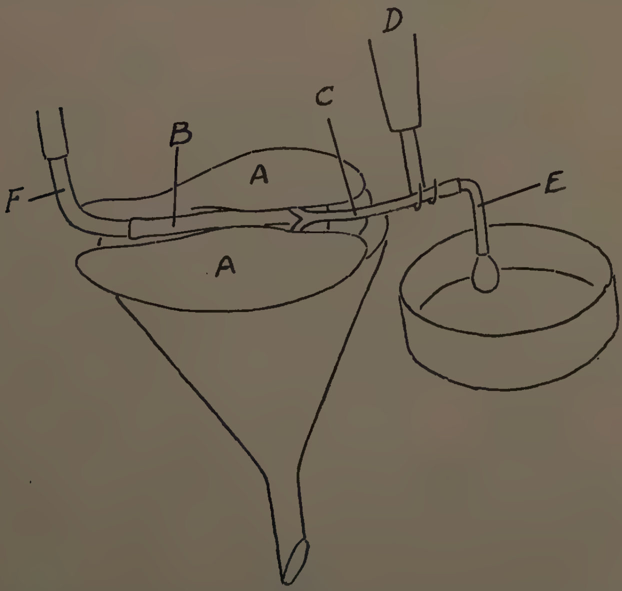

FIGURE 3. Diagram showing a preparation of the posterior salivary glands of *Octopus macropus* during perfusion and electric stimulation of the common duct. *A* are the posterior salivary glands; *B* is the aorta; *C* is the common duct; *D* are the electrodes; *E* is the platinum cannula; and *F* is the glass cannula into the aorta.

the addition to the perfusing fluid of substances such as tyramine, 5-HT, or acetylcholine.[15,17] Tyramine and 5-HT have a depressive effect; acetylcholine accelerates the flow. Only when the duct is stimulated mechanically or electrically is a liquid obtained, which is chemically and physiologically the same as normal saliva.[18] There is no spontaneous secretion.

Properties of Saliva

The saliva from *O. vulgaris* is extremely toxic for Crustacea. It is a very viscous liquid containing all the substances already mentioned[19] and, in addition, proteolytic enzymes[20] and a hyaluronidase.[21] Its powerful action upon the isolated heart of mollusks is chiefly due to the presence of 5-HT.[22,23,24]

When a drop of saliva is injected into a crab, the legs immediately contract and the animal remains in this position for about 1 min. The righting reaction is abolished, the appendages begin to tremble, the pincers open and close without external stimulation, and tetanic spasms of the body at intervals are seen. Little by little the animal becomes quiet; spontaneous movements as well as provoked reactions disappear and the aggressive animal of a few minutes before is changed to an inert limp organism. The heart beats as before and no apparent modification of the blood or of its circulation has occurred. The saliva has produced first a phase of overexcitability followed by a quiet phase and then paralysis.

A variation of this reaction follows use of an acetone extract of saliva. After removal of the acetone under reduced pressure, the residue is dissolved in sea water and injected into a crab. During the first minutes following the injection the animal shows all the symptoms of overexcitability described above, but the inert, quiet stage and paralysis never occur. After a few minutes the crab recovers completely. Chemical and chromatographic analyses show that the acetone extract contains all the active amines of the crude saliva.[19] It is well known that the blood of Crustacea has a pseudophenolasic activity,[25,26] and their organs, chiefly the kidneys and the hepatopancreas, are rich in amino oxidases,[22] so that the active amines can be readily inactivated by oxidation or oxidative deamination in the blood and tissues of the crab.

Equivalent amounts of pure tyramine, 5-HT, or octopamine produce the same effects as acetone-extracted saliva.

It is obvious to conclude from these observations that the saliva of Cephalopoda contains discrete factors responsible for the separate phases observed during poisoning of Crustacea. This is supported by the old experiments of Livon and Briot,[2] which we have confirmed. No excitatory reaction is produced by injecting a saliva that has been dialyzed against water: only standstill and paralysis result. No lethal effects are observed by injecting a saliva that has been heated for 10 min. at 100° C.; only the typical reactions due to overexcitability occur.

A substance having the same paralyzing action as dialyzed saliva has now been extracted and purified. It has been named cephalotoxin. Although the chemical composition of cephalotoxin has not yet been clarified, I shall describe the method used for its purification, some of its chemical properties, and a preliminary account of its pharmacological effects.

Extraction and Purification of Cephalotoxin

After several attempts, a method has been worked out for preparing cephalotoxin (Ct) from both saliva and crude extracts of p.s.gl. The method depends on the species used, but the procedure is basically the same as that shown for the glands of *O. vulgaris* in FIGURE 4.

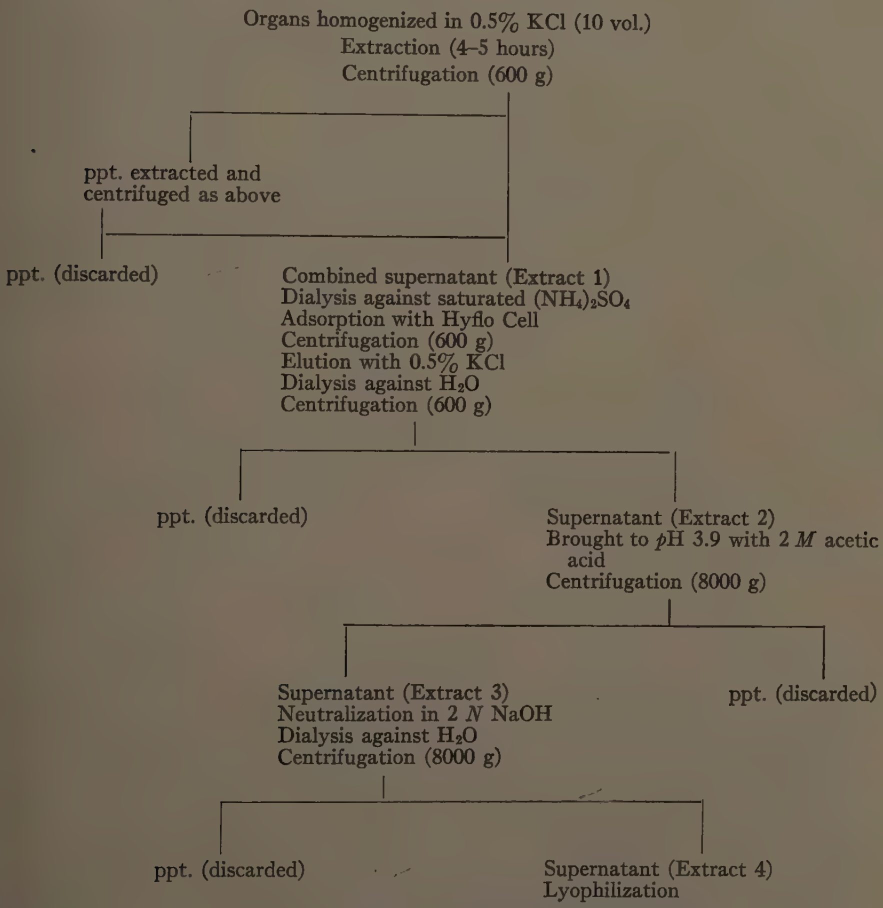

FIGURE 4. The extraction and purification of cephalotoxin from the posterior salivary glands of *Octopus vulgaris*.

The organs are homogenized in a Waring Blendor with 10 vol. of cold 0.5 per cent KCl, extracted for four hours in the cold with continuous stirring, and centrifuged at 600 g for 45 min. The sediment is resuspended in 10 vol. of the KCl solution, re-extracted, and centrifuged as before. The new sediment is discarded and the combined supernatants are dialyzed against cold saturated $(NH_4)_2SO_4$ for 24 hours. The precipitate is treated with a Hyflo Cell, centrifuged at 600 g, dissolved with 0.5 per cent KCl, and centrifuged again. The supernatant is dialyzed against water for 24 hours and the little precipitate formed is discarded. The supernatant is brought to pH 3.9 with 2 M acetic acid. A large precipitate is formed that is centrifuged off at 8000 g. The supernatant is neutralized with 2 N NaOH, and the new precipitate is also discarded. The clear supernatant is dialyzed against cold distilled water for 24 hours, and a little precipitate forms that is discarded by centrifugation.

At this step the extract may be lyophilized; a white, very light powder is obtained. This is very soluble in water or saline and can be kept in a desiccator at low temperatures for months. From 100 gm. of glands (fresh weight) 0.5 to 0.6 gm. of powder is obtained.

A similar method has been used for extracting Ct from saliva. In order to solubilize the extremely viscous saliva of *O. vulgaris*, it is diluted with three volumes of 15 per cent KCl and dialyzed againstm $(NH_4)_2SO_4$.

An arbitrary unit of activity is the amount of toxin that paralyzes a 40 to 50 gm. crab in 15 min. Specific activity may be calculated as units per mg. of proteins. However there is an alternative method for the determination of the activity, based on the response of the isolated esophagus or isolated aorta of *O. vulgaris*. These organs are very sensitive to Ct and the response is directly proportional to the amount of substance used. I shall refer to this later.

Table 1

Analysis of Cephalotoxin

Preparation No. 6 from the Posterior Salivary Gland of *O. vulgaris*

	(%)
Proteins (N determination)	74.05
Proteins (biuret reagent)	64.25
Carbohydrates	4.71
Hexosamines	5.80
Fructose	0
Pentoses	0
Uronic acids	0

When redissolved in water, Ct gives a positive biuret and ninhydrin reaction and an ultraviolet absorption spectrum with a maximum at 276 to 278 $m\mu$. After treatment with trypsin at 37° C. at pH 8.2, there is a complete loss of activity. Electrophoresis on starch gel has been done with Ct from *Sepia officinalis* (the cuttlefish).[27] At pH 8.5 four bands are observed migrating towards the cathode. Further purification of this preparation has been achieved by adsorption on calcium phosphate gel or on alumina Cy at neutral pH. After electrophoresis three bands are observed. They disappear after treatment of the substance with trypsin.

The results of an analysis of Ct from *O. vulgaris* are reported in TABLE 1. The values obtained for proteins were substantially different depending on the method used. The Folin reagent gives much higher values than the Kjeldahl method. Lower values are obtained with the biuret reagent. This may indicate the presence in Ct of high amounts of phenolic compounds. They were determined colorimetrically as tyrosine.

Ct was found to contain some sugars. No fructose, pentoses, or uronic acids are revealed by usual colorimetric reactions, but the reaction for hexosamines is positive. Hexosamines were determined colorimetrically after acid hydrolysis of Ct and adsorption of the interfering substances on Dowex. However, chromatography shows that the hexosamine present in Ct is not glucosamine.

Ct has no cholinesterase or aminoxidase activity. Moreover, a crude enzyme preparation from the electric organ of *Torpedo* as well as an aminoxidase preparation from Crustacea are not affected by the addition of Ct.

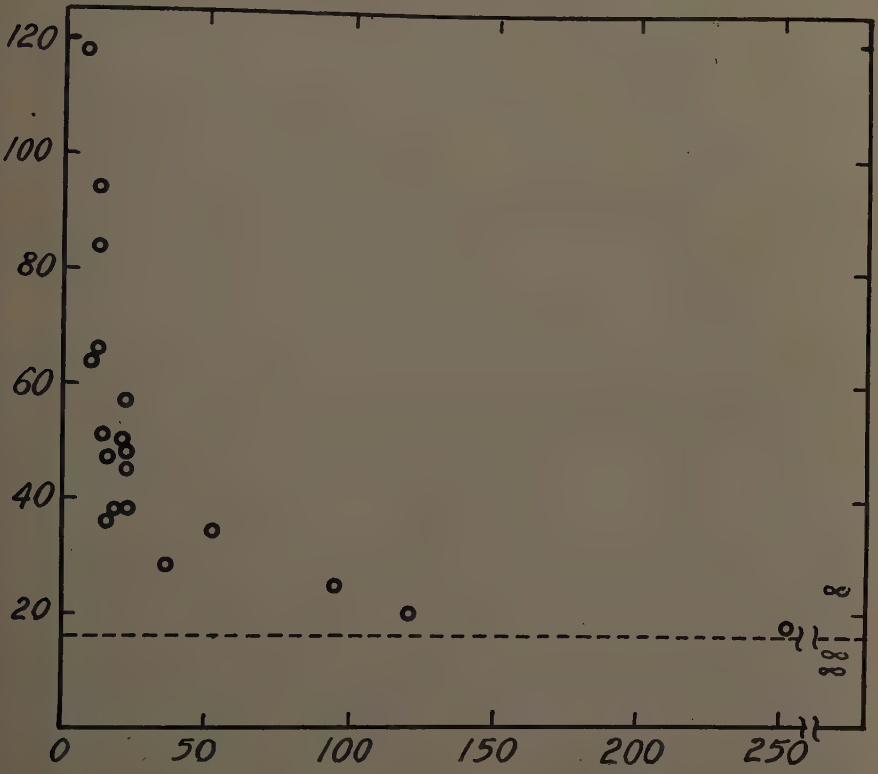

FIGURE 5. Time/concentration curve of cephalotoxin obtained with *Maja verrucoas.* The abscissa is the time in minutes between injection and paralysis; the ordinate is the concentration of Ct in mg./kg. of body weight.

Pharmacological Properties of Cephalotoxin

Injection of 0.1 ml. of a solution containing 0.2 to 0.4 mg. of Ct into a crab produces the reactions indicated when dialyzed saliva is used.

Different Crustacea are not equally sensitive to Ct. An amount of 0.1 mg. of substance per gm. of tissue produces complete paralysis in about 9 min. in *Eriphia*, whereas it takes 28 min. in *Maja* and 65 min. in *Squilla*. In *Palinurus* and *Pagurus* even longer times are required. Ct is also poisonous for insects. Bettini, of the Istituto Superiore di Sanità of Rome, has found (personal communication) that *Periplaneta americana* is sensitive to Ct, but it is necessary to use higher doses than for Crustacea.

The time between injection and paralysis depends on the amount of Ct injected. In FIGURE 5 is shown the time/concentration curve obtained with *Maja verrucosa*. The abscissa is the time in minutes between injection and paralysis and the ordinate expresses the concentration of Ct in mg./kg. body weight. The asymptote (indicated by dotted line) corresponds to the effective lowest concentration at an infinite period of time. It may be seen that in *Maja* the relationship between concentration of Ct injected and the toxic effect is a typical time/concentration curve. It has been calculated according the equation $(C - C_\infty)^n \cdot t = K$, which has given $(C - C_\infty)^{0.88} \cdot t = 381$.

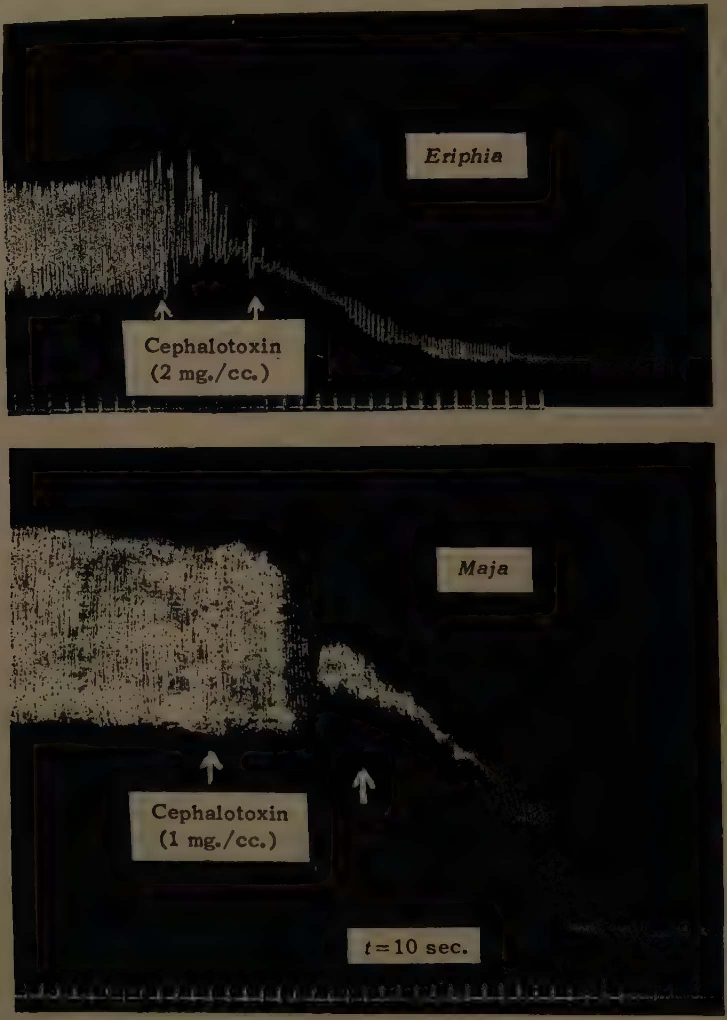

FIGURE 6. Action of cephalotoxin upon the isolated heart of two Crustacea, *Eriphia spinifrons* and *Maja squinado.*

Neuromuscular preparations of the pincers and legs of Crustacea are not affected by Ct. They promptly react to electric stimulation of their motor nerves after the animal or the preparation has been treated with Ct. Supra- and subesophageal ganglia in the paralyzed animal were also stimulated. No modification in the excitability was observed. Ct has no effect also on lobster neuromuscular synapses (H. Grundfest, personal communication).

Isolated hearts of *Maja* and *Eriphia* have been perfused with sea water containing Ct. At concentrations of 5×10^{-4} and 2×10^{-3} gm./l. the substance causes a temporary increase in tonus followed by decrease in amplitude and arrest in diastole (FIGURE 6). We have found the same reactions with the isolated central heart of *Octopus*. At 5×10^{-4} Ct has a small but continuing effect on the contractions, whereas at higher concentrations (2×10^{-3}) the heart always stops in diastole (FIGURE 7). In both cases the reactions are irreversible. Tyramine, 5-HT, or proteins such as fibrinogen or serum albumin, have an entirely different effect upon the heart. I should point out that these effects on isolated hearts can only be achieved with concentrations of Ct that are far higher than those which have a poisoning effect on the whole crab.

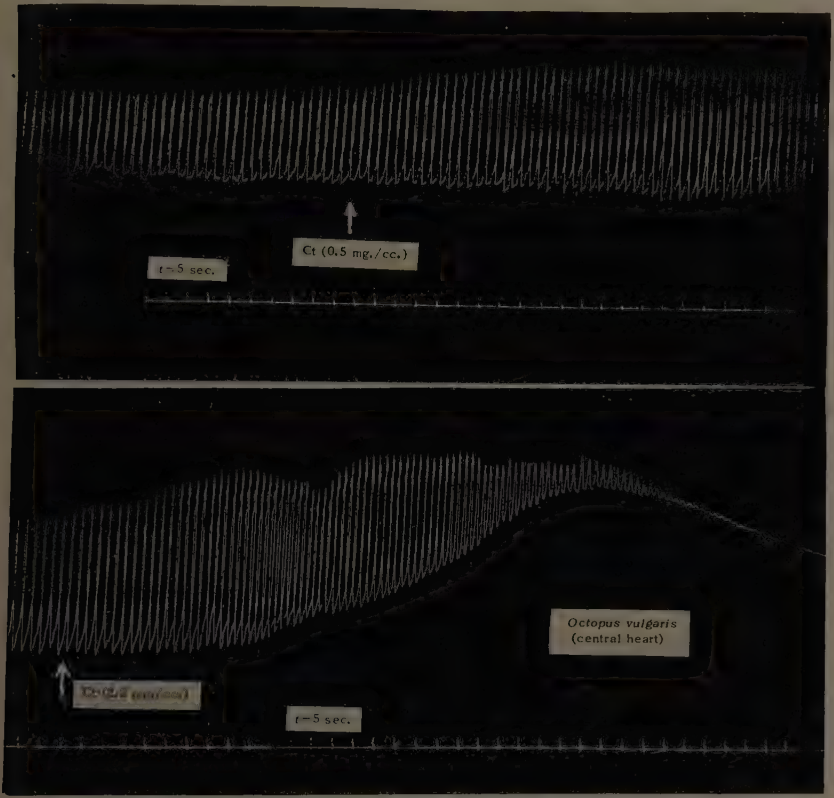

FIGURE 7. Action of cephalotoxin upon the isolated central heart of *Octopus vulgaris*.

The esophagus and aorta of *Octopus* have a rhythmic spontaneous motility

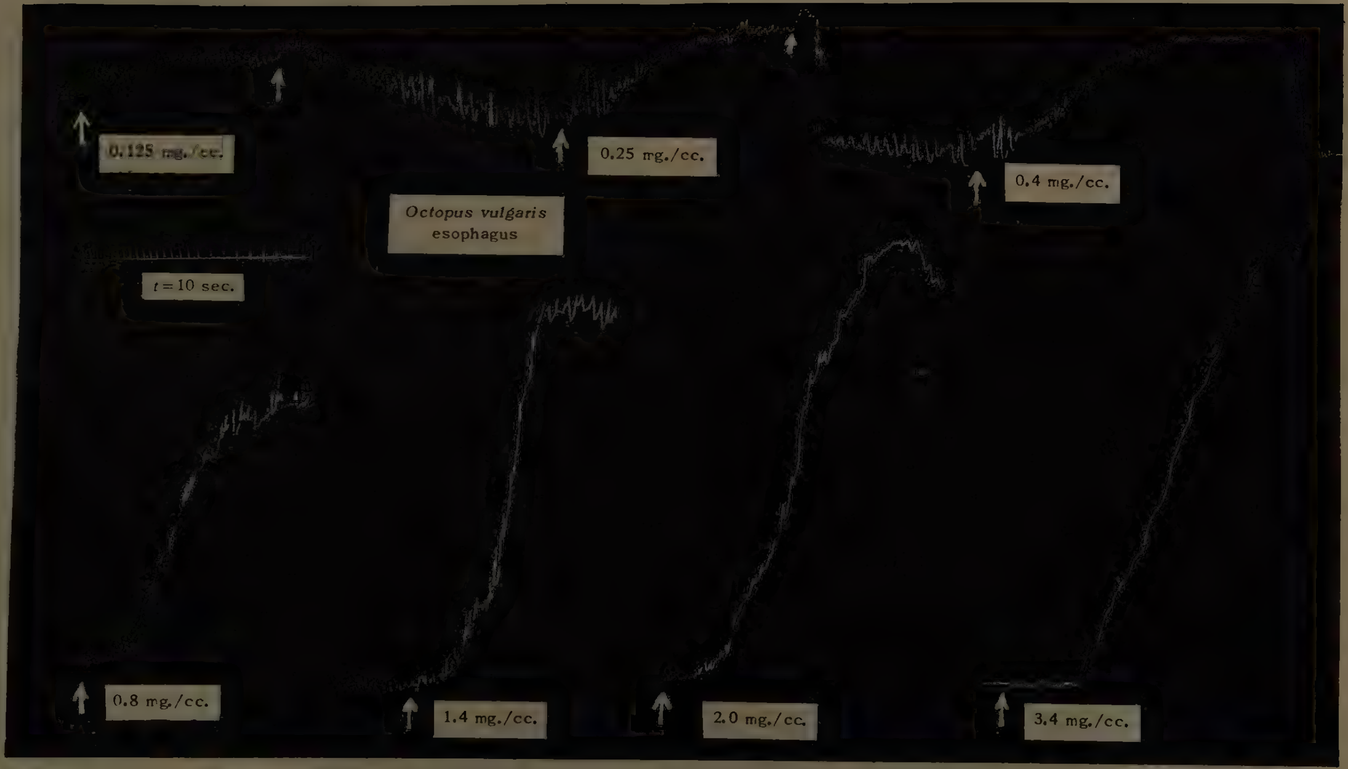

FIGURE 8. Response of the isolated esophagus of *Octopus vulgaris* to different amounts of cephalotoxin.

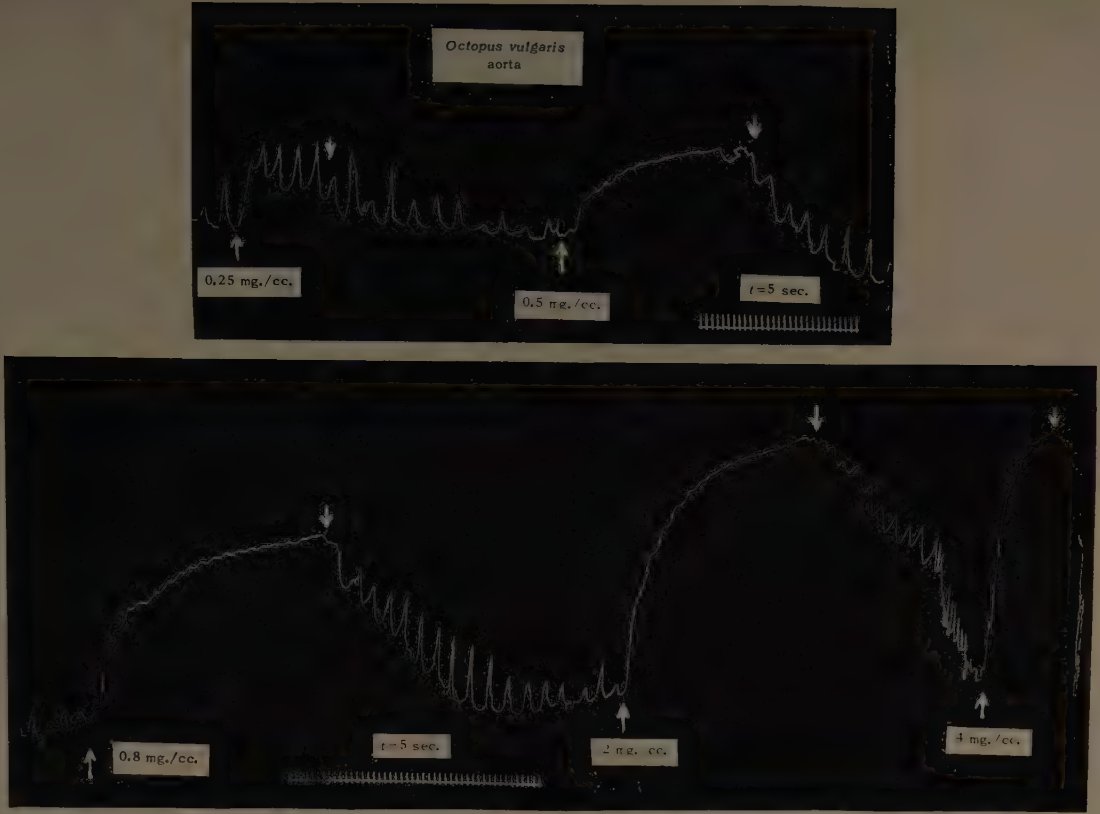

FIGURE 9. Response of the isolated aorta of *Octopus vulgaris* to different amounts of cephalotoxin.

that differs greatly from one animal to another. Addition of cephalotoxin to the sea water bath consistently causes an increase in tonus and in frequency of the spontaneous contractions. These effects are directly proportional to the amount of Ct used, and are abolished by washing (FIGURES 8 and 9). The *Octopus* esophagus and aorta have, in fact, been found very useful for an activity test of Ct. Both preparations give an average value of LD_{50} of about 0.33 mg./ml.

The action of Ct has been studied on a number of animals of different species and on isolated organs: the frog neuromuscular preparation; the frog esophagus and intestine; and different regions of the isolated gut of rabbit. The reaction of the isolated duodenum of a rabbit to Ct is shown in FIGURE 10. The organ is suspended in a Tyrode solution at 37° C. Addition of Ct (6.25×10^{-4}) causes an increase in tonus and amplitude of spontaneous contractions. The effect is abolished by washing.

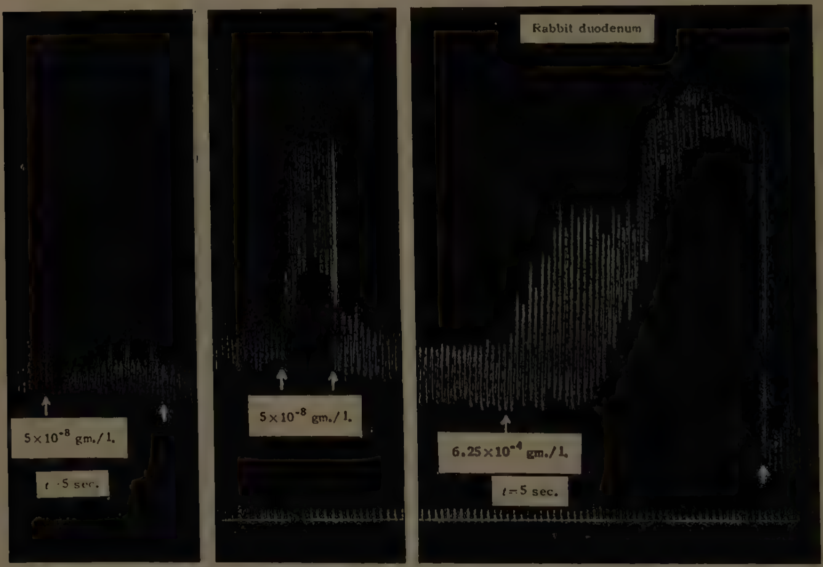

FIGURE 10. Action of cephalotoxin upon the isolated duodenum of the rabbit.

Ct has a strong inhibiting effect upon respiration of Crustacea. This action has been followed manometrically on small crabs (*Carcinus* and *Pachygrapsus*) and by Winkler's determination of oxygen remaining in sea water in which larger animals, such as *Maja* and *Squilla*, were kept. Some of the results are reported in FIGURE 11 and TABLE 2. It may be seen that, after injection of Ct, the oxygen consumption drops rapidly and an inhibition of about 70 per cent is observed. No modification of the respiration of isolated organs (gills), tissues (muscles), or tissue homogenates (hepatopancreas) can be detected after addition of Ct or by comparison between normal and paralyzed animals. The substance has no effect upon the cytochrome oxidase activity of mito-

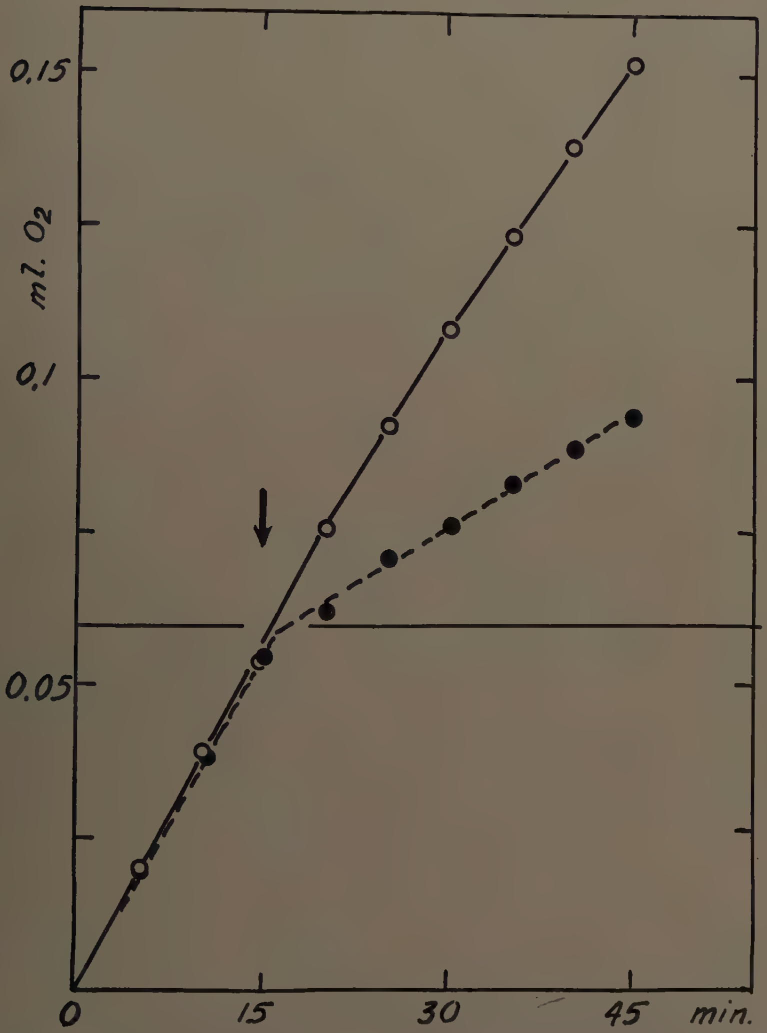

FIGURE 11. Effect of cephalotoxin on the respiration of *Carcinus maenas*. At the time indicated by the arrow, Ct was added. The abscissa represents time in minutes; the ordinate represents ml. of oxygen uptake. The open circles indicate the control animal and the dark circles represent the cephalotoxin-treated animal.

TABLE 2
ACTION OF CEPHALOTOXIN ON THE RESPIRATION OF CRUSTACEANS

	Q_{O_2}		inhibition (%)
	normal	treated	
Carcinus	109	34	69
Carcinus	98	29	70
Carcinus	106	28	73
Pachygrapsus	112	36	68

chondria prepared from the body muscle of crabs. The mechanism of this inhibition of respiration observed with entire animals is unknown.

It has been found that Ct inhibits blood coagulation of Crustacea. For these experiments lobster blood was used. Heated plasma and lobster muscle extract have been also prepared and clotting time was determined in the presence of different amounts of Ct. It is generally accepted that two mechanisms are active in hemostasis in Crustacea, namely an agglutination of the blood cells and a coagulation of the blood plasma. Inhibition of both mechanisms occur in the presence of Ct.

Conclusions

As may be seen, the study on Ct is at its very beginning. What has been done until now has furnished some indications that allow us to draw no definite conclusions on either the chemical nature of the substance or the mechanism of its activity against Crustacea. More work has to be done to find an answer to these questions.

Experiments have been undertaken to prove that Ct is a glycoprotein and eventually to establish its active group. Ct from different Cephalopoda shows the same properties against Crustacea, but we have no evidence that the substance always has the same chemical composition. Some of Ct's pharmacological properties have been established; however the physiological mechanism of its activity is unknown. Is Ct specific for Crustacea or, under certain circumstances, can it also be active against other groups of invertebrates and vertebrates? As a matter of fact, the blood coagulation is strongly inhibited also in man, but only experiments of the most preliminary kind have been done on this subject. Ct seems to be able to modify the electrical activity of the central nervous system of crabs. Experiments are in progress to establish if one may find there the keystone of the entire problem.

Acknowledgments

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THE TOXIN OF *PHYSALIA* NEMATOCYSTS*

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It is basic to any discussion of the pharmacology and biochemistry of the nematocyst toxin of *Physalia* to review the structure of the animal and of the organoids within which this toxin is contained. The function of these structures in the normal life of the parent organism should also be considered.

Dependent from the pneumatophore of *Physalia* is an assemblage of polyps of different kinds. Outstanding among these are the dactylozooids, or fishing tentacles. In an adult *Physalia* the fishing tentacles may extend 100 feet in length. Coaxial with the dactylozooid and running along its entire length is a band of specialized tissue that covers diverticulae of the gastrovascular cavity of the tentacle. When the tentacle contracts it shortens more completely than this superficial structure, which causes the latter to be thrown into loops and folds referred to as "batteries." The nematocysts are contained in cnidoblasts located in the superficial epithelium of the battery. The toxin to be described in this paper is contained as a structureless fluid within the nematocyst capsule. In life it fills the globular cavity and bathes the surface of the nematocyst tubule.

There are two general sizes of nematocysts: the smaller is about 9 μ in diameter, and the larger is approximately 3 times as large. There may be functional differences between these classes of nematocysts, but our studies have not revealed them. Nematocysts are distributed throughout the thickness of the battery epithelium: the larger ones occupying the entire thickness of the epithelium and the smaller ones being symmetrically disposed between the larger ones both horizontally and vertically.

The mature nematocyst nearly fills the cnidoblast within which it is produced, pushing the now-pyknotic nucleus to one side of the almost structureless cytoplasm. The globular capsule wall appears to be invaginated at one point. The highly coiled internal tubule retains its connection with the capsule wall at this point. What would have been an open connection between the lumen of the internal tubule and the cytoplasm of the cnidoblast is closed by the operculum. A cnidocil or trigger protrudes a few microns into the ambient water.

In nature the fishing tentacles are in continuous restless motion, alternately relaxing and contracting, constantly sampling the water beneath the pneumatophore. When the tentacle brushes against a prey organism, whether it is planktonic crustacean or larger fish, the cnidocil of superficial nematocysts are stimulated and they trigger the immediate release of the coiled nematocyst thread. When fully uncoiled, this may be several hundred times as long as the diameter of the parent capsule. The tubule is armed thoughout its length with chitinous barbs and spines and constitutes an extremely effective "tangle." If the tip of the nematocyst thread should penetrate the prey organism, this hol-

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This paper is Contribution 279 from the Marine Laboratory of the University of Miami.

low threadlike tube conducts the nematocyst toxin directly into the body of the prey. In our laboratory it has been observed frequently that the nematocyst thread can penetrate even a surgical glove. It should be emphasized that the magnitude of the response to contact with the prey organism is proportional to the area of contact between tentacle and prey. It is conceivable that a single copepod might elicit the discharge of 20 to 50 adjacent nematocysts, while contact between the tentacle and the side of a fish would discharge hundreds of thousands of nematocysts. Initial gentle stimulation of the nematocyst probably evokes rapid release of the nematocyst thread but does not dislodge the parent capsule from its position within the epithelium. Vigorous resistance of the unsubdued prey greatly increases the numbers of nematocyst involved in the reaction and dislodges many from the epithelium. These are replaced by cnidoblasts that differentiate outside the battery and, secondarily, come to occupy a definitive position in the battery epithelium.

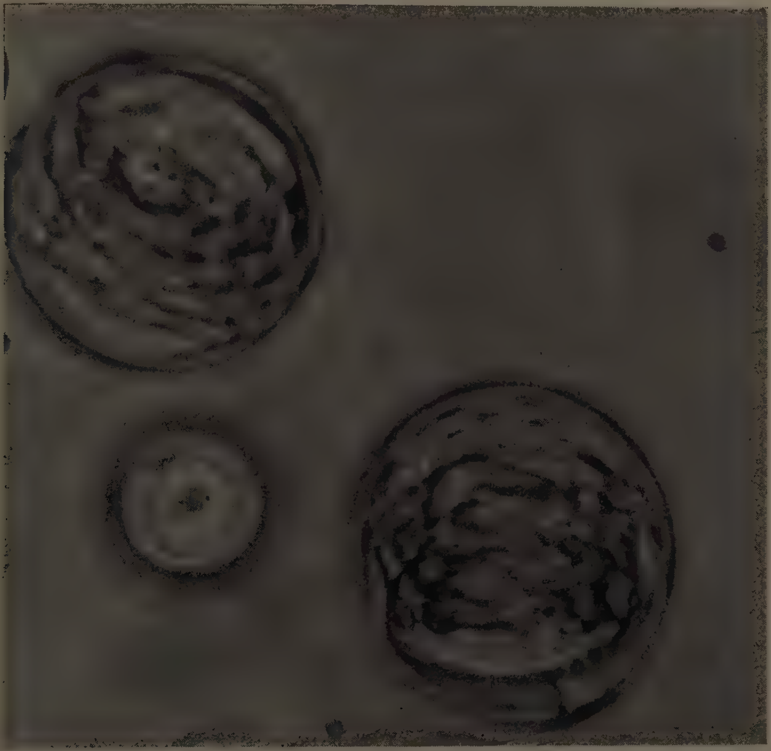

FIGURE 1. Undischarged nematocysts separated from tentacular tissue. The coiled internal tubule and the operculum of the larger nematocysts are noteworthy. Examples of both size groups of nematocysts are shown. ×1600.

The mechanism by which the internal capsular thread uncoils and propels itself into the ambient water is still obscure. Indeed, even the nature of the adequate stimulus for discharge is uncertain. By whatever means the process is initiated its course is fairly clear: there is a rapid eversion of the tubule that, in its essence, resembles the eversion of the finger of a glove. The process is fairly rapid but could hardly be accurately described as "projectile" since it requires several seconds to reach completion *in vitro*. There is a suggestion, from field observation and experience, that this reaction may be more rapid *in vivo*.

The tissues of the tentacle contain biologically active materials in addition to the toxin that will be described here. Thalassin and congestin (Richet, 1903) almost certainly originate from general tentacular tissues outside the nematocyst. It appears unlikely that brief grinding of the whole tentacle with sand would produce a significant breakdown of nematocysts. The "*Cyanea* principle," effective in causing release of histamine from mast cells, described by Uvnäs elsewhere in this monograph, is apparently also of tentacular origin. In this connection it may be of interest that samples of *Physalia* toxin from this laboratory have been inactive in eliciting histamine release from mast cells in Sweden. To clarify the pharmacology of the nematocyst and to insure that the toxin to be described could have originated only from the nematocysts, the

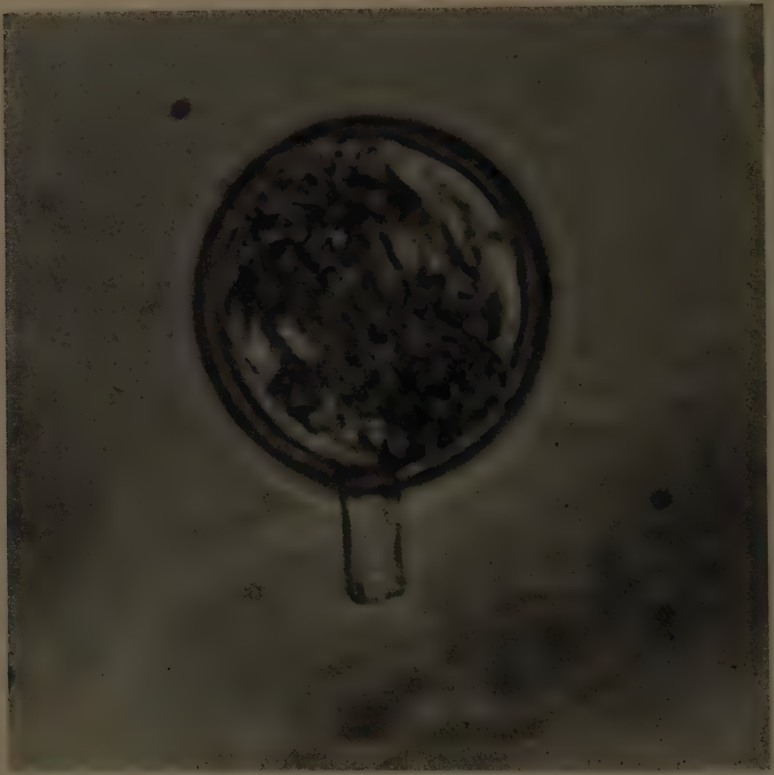

FIGURE 2. The beginning of discharge of isolated nematocyst. The hollow tubule is clearly visible. ×1600.

following method was employed to separate still-reactive nematocysts from other tentacular materials (Lane and Dodge, 1958).

The tentacles are removed from living, freshly stranded *Physalia* in the field. They are freed of adherent debris and seawater by decantation. The tentacle tissue is returned to the laboratory and autolyzed for 24 hours at refrigerator

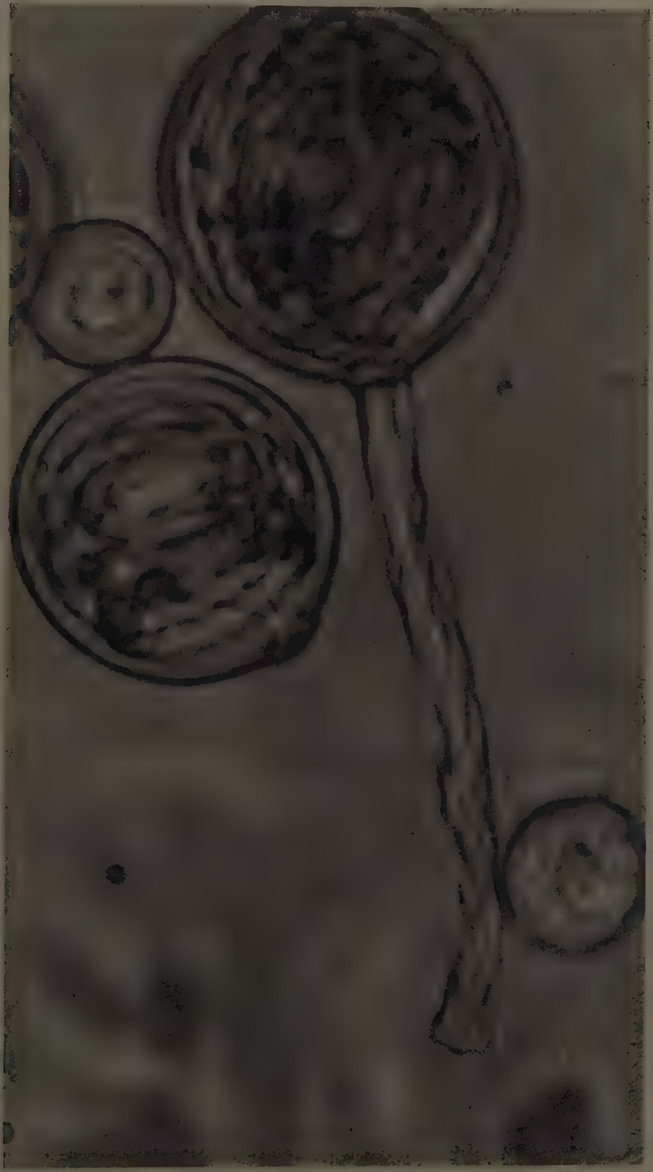

FIGURE 3. Later stage in the discharge of isolated nematocyst. The connection between the internal tubule and operculum appears in the undischarged large nematocyst in this figure. ×1600.

temperatures. After this period has elapsed, the tentacle material is forced through graded sieves of up to 40 mesh/inch to hasten the breakdown of the tissues and to accelerate the liberation of the nematocysts. The sieved brei is suspended in clean sea water and returned to the refrigerator. Autolysis proceeds rapidly and, within 36 hours, the nematocysts begin to separate from other components in the container. They do not tolerate centrifugation and are allowed to settle out by gravity. Contaminating autolyzed tissue components and other soluble materials extraneous to the nematocysts are decanted from the nematocyst layer, settled nematocysts are resuspended in additional clean sea water, and are again permitted to settle. This process of alternate suspension and settling is continued until the wash water, which initially contains considerable biological activity, is no longer toxic to *Uca* when injected into the hemocele. The washed nematocyst concentrate assumes the appearance and the consistency of freshly ground putty. Each gallon of tentaculur tissue yields 60 to 75 gm. of pure nematocysts. Representative preparations have averaged about 55 million nematocysts/gm. The washed and concentrated nematocysts may be frozen at −20° C. and stored in this condition for up to 3 years with no significant loss of reactivity to human skin.

The toxin is prepared by homogenizing the concentrated purified nematocysts in an all-glass, motor-driven homogenizer. Other preparative methods have been only partially successful in breaking down the wall of the capsule. It is necessary that the nematocyst capsule actually be broken to release the liquid contents. Mere discharge of the contained tubule does not liberate all the toxin into the ambient medium. The homogenate is freed of particulate debris by centrifugation at 12,000 g at 4° C. for 20 min. Alternatively, the particulate fragments might be adsorbed on calcium phosphate gel (Wangersky and Lane, 1960). After either treatment there remains a water-clear toxin solution, which is then lyophilized. The dried toxin is stored in evacuated capsules in the dark at −20° C. Under these conditions of storage, activity is undiminished druing 3 years. The dried toxin contains 15 to 16 per cent total nitrogen by micro-Kjeldahl Assay on different preparations.

When lyophilized toxin is reconstituted with physiological saline and injected into adult male Swiss mice of about 30-gm. weight, the certainly lethal dose level for the intraperitoneal route is 2.0 to 2.5 mg./kg.

After intraperitoneal injection the onset of intoxication is immediate. Pain, increased activity, and tremors appear at once. It is thought that these symptoms are produced by local irritation rather than by direct central nervous system stimulation. After 10 min., ataxia, decreased muscle tone, flaccid paralysis of the hind limbs, and depressed rate of breathing are characteristic of injected animals. Then follows urination, defecation, aphrodisia, marked myosis, dyspnea, cyanosis, anoxic convulsions, and death due to respiratory failure. Survival time is 1 to 48 hours, depending on the dose administered. Postmortem examination shows the following gross pathology: lungs blanched; heart contracted, especially the left ventricle; hemorrhagic edema into the peritoneal cavity; and skin, nose, and ears very white.

A dose level of 16.2 μg. of dried toxin dissolved in 0.5 ml. of frog Ringer's solution was uniformly lethal when injected into the ventral lymph sac of each

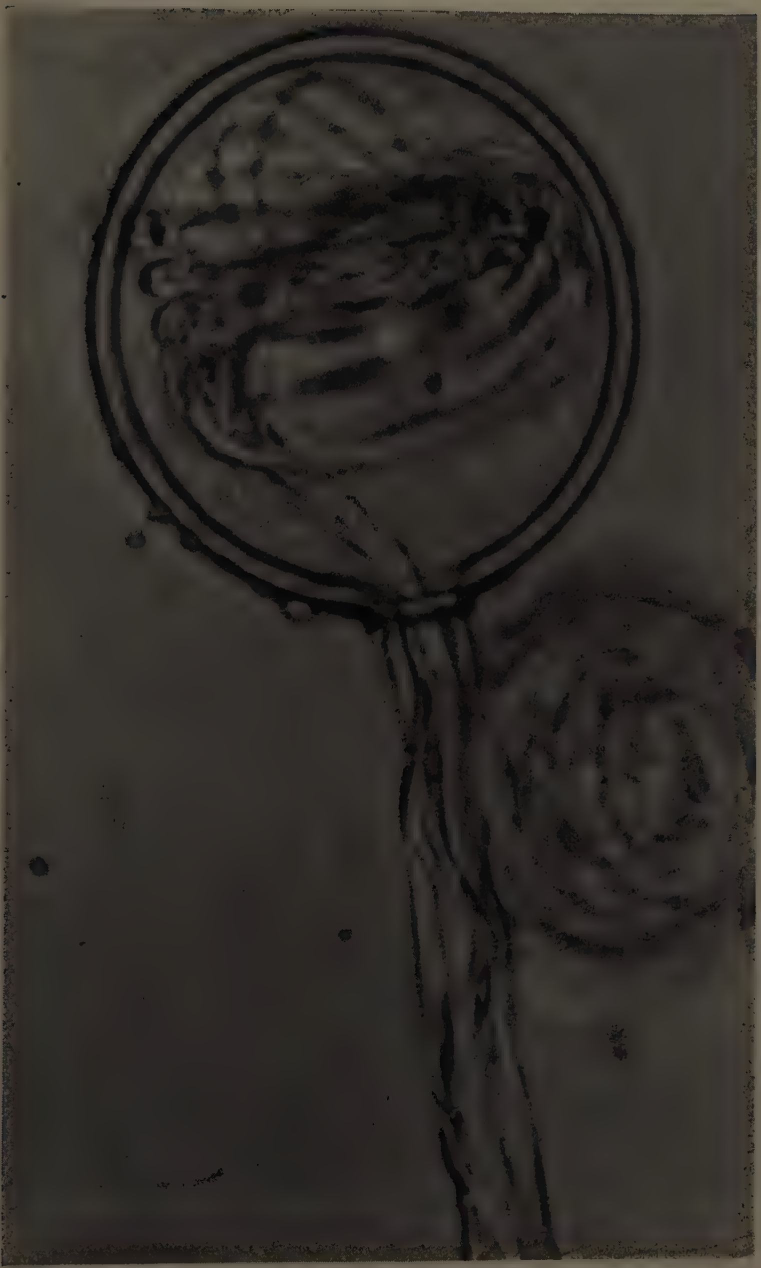

FIGURE 4. A discharging isolated nematocyst. The coiled internal tubule and the hollow nature and external architecture of the everted tubule are shown. ×2800.

of eight frogs (*R. pipiens*). There was an almost immediate appearance of localized dermal petechiae, most clearly visible on the white ventral surface. Breathing became rapid and shallow. Central nervous involvement was signalized by deterioration of the postural and righting reflexes. When spinal reflexes disappeared, electrical stimulation of the sciatic nerve produced no response, but direct stimulation of the gastrocnemius showed it to be still normally reactive. Heartbeat persisted for 12 to 24 hours. Large amounts of lymph accumulated in the subcutaneous sinuses. Viscera were hyperemic, bladder and intestine were empty, and considerable bloody peritoneal fluid was observed.

Intramuscular injection of adequate doses of crude toxin into fish is followed by immediate hyperventilation, disorientation, and spasmodic bursts of rapid swimming, alternated with prolonged periods of inactivity and death within one to four hours. Motor paralysis appears on the injected side. Sclerocorneal petechiae were frequently observed. At the site of the injection there was contraction of chromatophores, which caused a localized blanching. The general body surface away from the point of injection appeared to darken.

There was no hemolysis of erythrocytes of the mullet *Mugil cephalus* when they were incubated at 37° C. with several dilutions of crude toxin.

The toxin produced irreversible damage in the isolated heart of the clam *Mercenaria campechiensis*, which prevented duplication of response by subsequent treatment of the same preparation with similar dose levels. The initial response was reminiscent of that produced by the administration of acetylcholine, that is, diastolic arrest.

Various levels of crude toxin, when tested auxanographically with ten species of marine yeasts and seven species of marine bacteria, have been without effect. The toxin appears not to influence either survival or growth and reproduction of *Paramecium caudatus* or of *Tetrahymena pyriformis* (unpublished observations made by Bradner W. Coursen in this laboratory).

A copious precipitate is produced by mixing crude *Physalia* toxin with the plasma of the loggerhead turtle *Caretta caretta*. It was thought (Wangersky and Lane 1960) that this precipitin reaction might be invoked to explain the observed ability of the loggerhead turtle to ingest large numbers of *Physalia* without apparent damage. However, when the precipitate was removed by centrifugation and the remaining combined toxin-plasma solution was assayed on mice, it was found that the original toxicity was in no way diminished. It therefore appears that this precipitate of plasma and toxin does not explain the apparent insensitivity of the loggerhead to the toxin of the *Physalia* on which it often feeds.

Lyophilized toxin is stable for at least 3 years in evacuated capsules held at −20° C. in the dark. The toxin is thermolabile in solution, its activity being completely destroyed during five minutes at 60° C. Reaction with ethanol, acetone, diethyl ether, petroleum ether, and most other organic solvents destroys the activity of crude toxin preparations.

The crude toxin is nondialyzable and strongly biuret-positive. Benedict's, Molisch's, and anthrone tests are all negative, which suggests little or no polysaccharide present. One-dimensional descending paper chromatography with

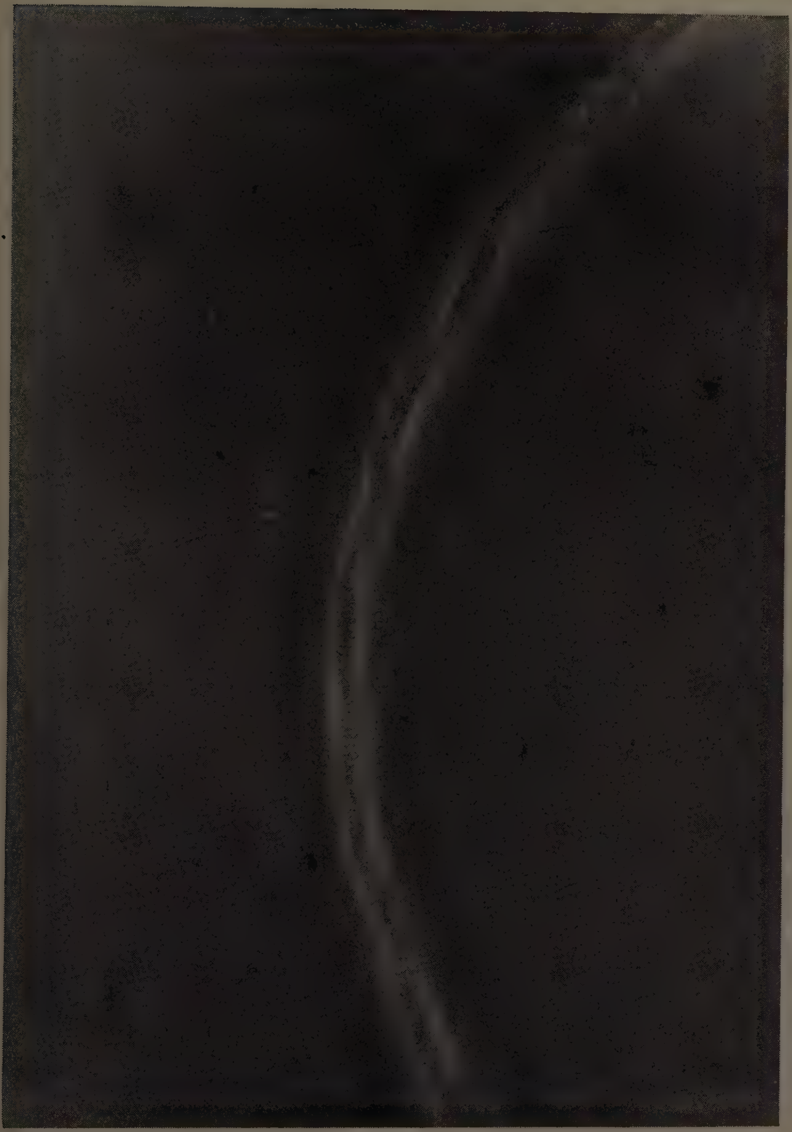

FIGURE 5. Tubule of discharged nematocyst photographed with dark field illumination. The helical bands of chitinous spines appear as rows of white dots. ×2800.

80 per cent *n*-propanol as the solvent system has clearly separated the crude lyophilized toxin into nine components. Subsequent elution, acid hydrolysis, and rechromatography of each of these zones has shown them to be peptides, of qualitatively and quantitatively different amino acid composition. Further study of the biological activity of these chromatographic fractions is one of the present objectives of this laboratory.

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MECHANISM OF ACTION OF A HISTAMINE-LIBERATING PRINCIPLE IN JELLYFISH (*CYANEA CAPILLATA*)*

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In the Department of Pharmacology of the Karolinska Institutet, we have for some years been interested in the mechanism of histamine release. This is not a new problem; the release of histamine, especially in anaphylaxis, has in fact engaged the attention of physiologists for decades. However, some observations in recent years have served to modify the problem. It has been found that histamine, or at least the mobilizable histamine—that released during allergic reactions—is localized in special cells: the mast cells. These are the same cells that contain heparin, a discovery made by Jorpes and his associates in Stockholm about 25 years ago.[7] It is generally agreed that the histamine—like heparin—is in some way or other stored in the basophilic intracellular granules of the mast cells.

Around 1940, substances of a new type were discovered, the histamine liberators.[8] These substances, which include several well-known drugs, have a more or less specific ability to release histamine in the mammalian organism.

It was soon observed that morphological changes occurred in the mast cells when histamine was released by histamine liberators and by antigens. When such "emptied" cells are fixed in formalin and stained with toluidine blue, the basophilic granules can be seen scattered around the cells. It has since been demonstrated that these morphological changes reflect in some way or other the release of histamine and other biologically active substances from the cells.

The discovery that the releasable histamine is localized in the mast cells nullified the prevailing theories on the mechanism of histamine release. According to previous more or less diffuse theories, histamine was bound to tissue proteins or lipoproteins, from which it was released enzymatically. In most theories proteases were assumed to take part in this histamine release. The experimental approach has now changed and poses the question: How is the histamine released from the mast cells? It was this question that my associates and I took up in Stockholm, and it led to our interest in *Cyanea capillata*, the stinging jellyfish.

Cyanea capillata (FIGURE 1) occurs along the Swedish west coast and may be a great annoyance to bathers during late summer and early fall. Contact with its tentacles causes a burning sensation with itching and pain, redness of the skin, and sometimes local edema. Especially distressing are the skin symptoms if the tentacles strike the face.

It seemed to us fairly plausible to assume that histamine was in some way the causative agent behind the skin reactions produced by contact with the tentacles. In principle, two possibilities had to be considered. The nematocysts might contain histamine, as do the hairs of the stinging nettle. On contact with the nettle hair, the end of the hair is broken off and its histamine is

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introduced into the skin. In the same way the nematocysts could be thought to contain histamine that would enter the skin when the nematocysts burst. The other possibility was that the nematocysts contained a histamine liberator that, on introduction into the skin, causes local histamine release from the skin mast cells.

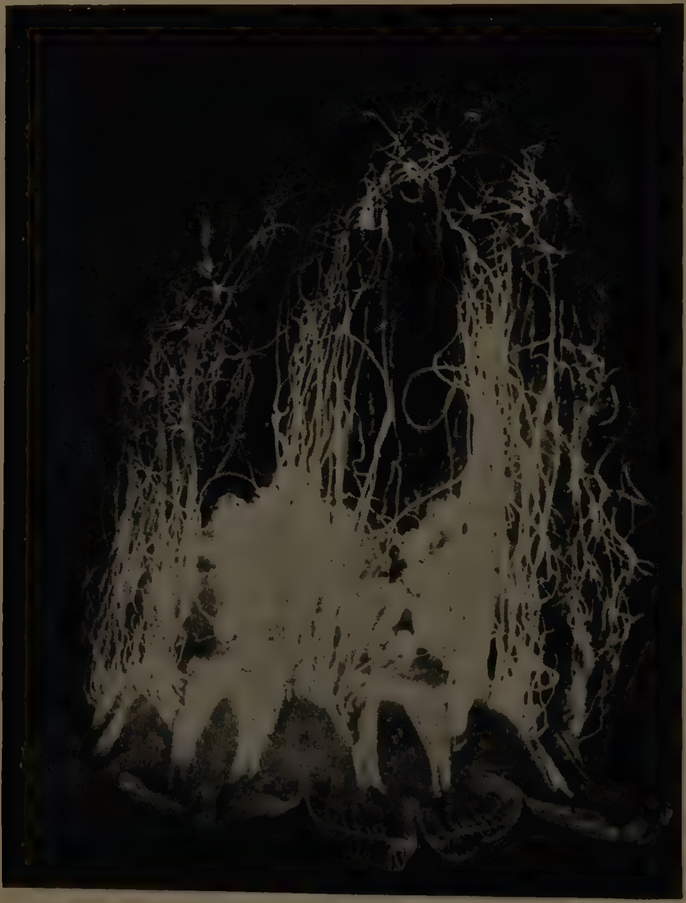

FIGURE 1. *Cyanea capillata.*

If the tentacles contained an extractable liberator, it might be possible to get a suitable model substance for the study of the mechanism of histamine release. Such naturally-occurring histamine liberators might act like antigens. Histamine liberators should also be better suited for quantitative studies of the histamine-release process.

Methods

Extracts from the tentacles showed no histamine, but we found a rather active histamine-releasing agent.

Cyanea tentacles were extracted for several weeks in 50 per cent ethyl alcohol. After a series of precipitations in alcohol of increasing concentrations, the active material was dissolved in hot absolute alcohol. The precipitate that formed when the alcohol was cooled showed a rather high histamine-releasing activity. This technique is, in principle, the one used by Richet[9] many years ago for isolating the toxin "thalassin" from sea anemones and other lower marine animals. A further purification of the raw material could be obtained by adsorption to charcoal or amberlite. Most of the observations described in this paper were made on alcohol-precipitated material.

Several methods have been used in our laboratory for the study of the histamine-releasing factor from *Cyanea*.

Cat skin has a high histamine content and is sensitive to several histamine releasers. The skin of the cat paw is especially rich in histamine, and we have developed a technique that permits the histamine-releasing activity of a liberator to be measured in the perfused paw.[11] With this technique, extracts from *Cyanea* tentacles were observed to contain an active histamine-releasing agent.[3] Since this histamine release was thought to be due to degranulation of mast cells in the skin, we turned to techniques that enabled us to study the action of liberators directly in mast cells.[2] The mast cells of rat mesentery are especially suited for microscopic observations, since the cell granules take up and retain basic stains such as toluidine blue even when they have spread outside the cell.

We have recently developed a technique by which it is possible to study histamine release from isolated rat mast cells.[13] These isolated cells can also be used for study of the morphological changes that accompany the histamine release (FIGURE 2). The isolation technique is in principle very simple. It is, in fact, a modification of a method developed by Glick in the United States.[16] An isosmotic salt solution is injected into the abdomen of a freshly killed rat. After a few minutes' light massage the abdomen is opened and the salt solution withdrawn by a drop pipette. The fluid contains peritoneal cells, some of which are mast cells. The cell-containing fluid is layered in a centrifuge tube above a polysaccharide solution of suitable specific gravity. On centrifugation at a suitable rate the mast cells pass down into the polysaccharide solution, and an almost pure mast cell suspension can be obtained. This suspension is freed from the polysaccharide by repeated washing with physiological salt solution.

The isolated mast cells can then be incubated with histamine liberators and antigen, and the influence of various factors on the histamine release can be studied.

Results

FIGURE 3 illustrates the influence of *p*H on the histamine release caused by the *Cyanea* factor. The *p*H range is fairly narrow, with the optimum between 6 and 7.

The histamine-releasing action shows temperature dependence. As is evident from FIGURE 4, histamine release does not occur at 0° C., but it increases with rising temperature to about 37 to 40° C. When the temperature exceeds 43 to 45° C. the release curve falls abruptly. Worth noting is the fact that the temperature effect below 37° C. is reversible; in other words, the release from cooled cells becomes normal when the temperature is increased to 37° C. On the other hand, the abrupt fall of the histamine release at temperatures

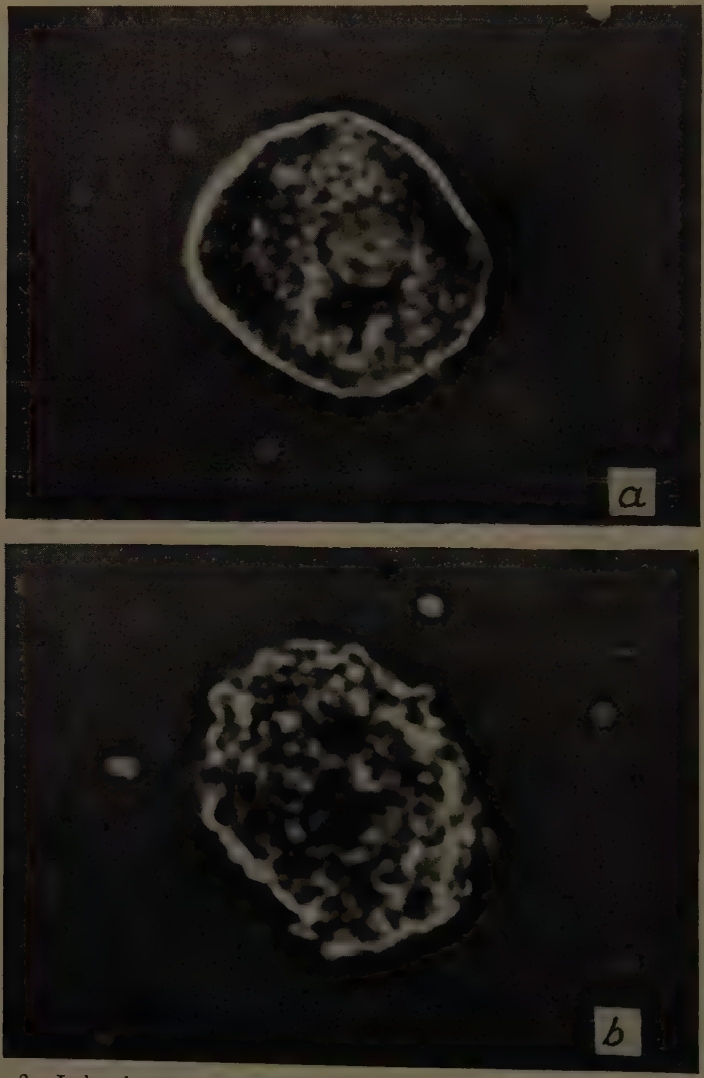

FIGURE 2. Isolated rat mast cell shown (*a*) before and (*b*) after addition of the histamine-release agent (microphotograph made with a water-immersion lens).

above 43° C. is irreversible. The release is not normalized on reincubation at 37° C. The *Cyanea* principle is rather heat stable. As I shall mention below, the course of the temperature curve is one of the indications that the histamine release results from an enzymatic process in the mast cell. The fall of the release curve on heating the mast cells might be attributed to inactivation of this enzymatic process, possibly by destruction of lipid structures. In our opinion the enzymatic process, possibly in its initial phase, may involve the inactivation of a phospholipase.[4,6,14]

We have studied several enzyme inhibitors that have been shown to block mast cell degranulation and histamine release produced by other histamine liberators[4,6,14,15] and by antigen.[12,13] The action of the *Cyanea* factor also is blocked by such inhibitors. We have studied the influence of some that are thought to block NH_2 and SH groups more or less specifically. FIGURE 5 illustrates the inhibitory action of ninhydrin—which is considered to block NH_2 groups—and FIGURE 6 shows the similar action of allicin, a substance considered to block specifically SH groups.[5] These two inhibitors in rather low concentrations completely block the histamine-releasing action of the *Cyanea* extracts. The blocking action of allicin can be counteracted by gluta-

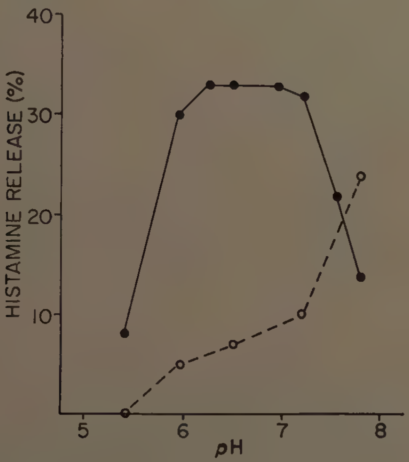

FIGURE 3. Influence of *p*H on histamine release by *Cyanea*. ●——● *Cyanea* 50 µg./ml. ○----○ Control.

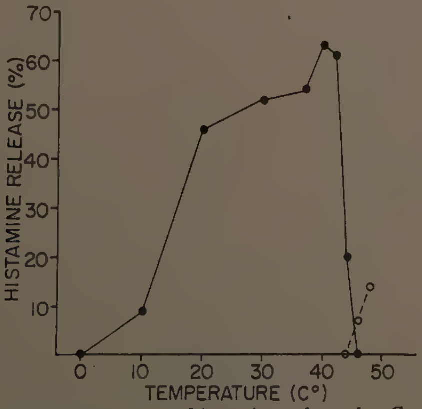

FIGURE 4. Influence of temperature on histamine release by *Cyanea*. ●——● *Cyanea* 50 µg./ml. ○----○ Control.

thione, a fact lending further support to the opinion that the inhibitory action is due to SH block.

From our observations on the influence of *p*H, temperature, enzyme inhibitors, and some other factors on mast cell degranulation and histamine release produced by various histamine releasers and by antigens, we have ventured to hypothesize that the histamine release from mast cells is due to the activation of an enzymatic process localized in the mast cell itself.[6,12-14] Space limitations do not allow me to discuss these problems in detail, but we have indirect evidence that at least one phase of the enzymatic process is the activation of a phospholipase. Of about 40 enzymes studied, only one was observed to degranulate mast cells; namely, phospholipase A.[4] We have found, too, that all

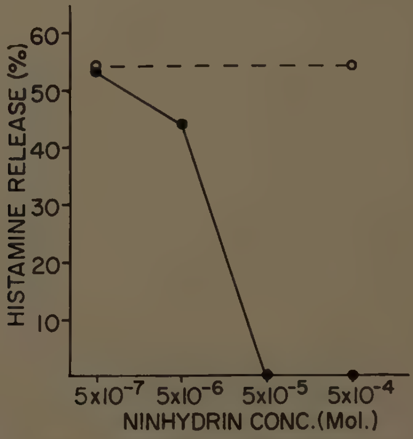

FIGURE 5. Inhibitory action of ninhydrin on histamine release by *Cyanea*. ●——● Ninhydrin + *Cyanea* 50 μg./ml. O----O *Cyanea* 50 μg./ml.

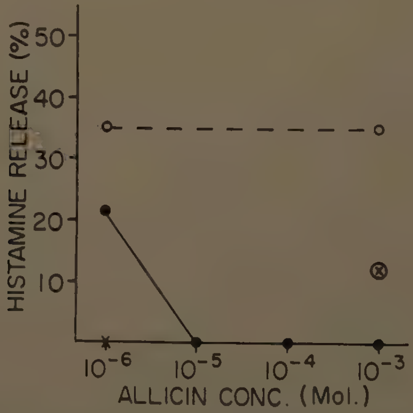

FIGURE 6. Inhibitory action of allicin on histamine release by *Cyanea*. ●——● Allicin + *Cyanea* 50 μg./ml. O----O *Cyanea* 50 μg./ml.

the enzyme inhibitors that block the action of our histamine liberators also block the degranulating action of phospholipase A. Thus far we have not come across any exception to this rule; in other words we have found no inhibitor that blocks the action of phospholipase A but not the action of histamine iberator, and vice versa.

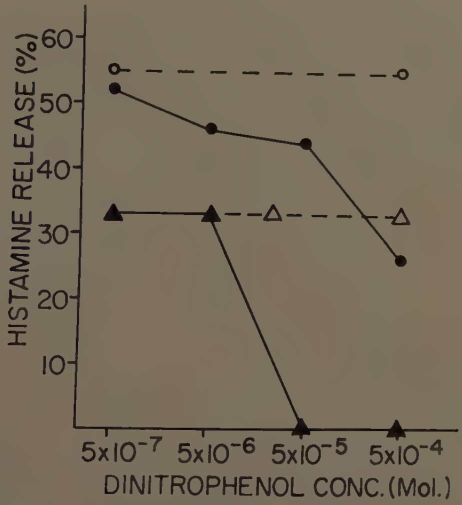

FIGURE 7. Influence of glucose on histamine release by *Cyanea* and on inhibitory action of dinitrophenol (DNP). ●——● *Cyanea* + dinitrophenol + glucose (0.2 per cent). ○- - -○ *Cyanea* + glucose (0.2 per cent). ▲——▲ *Cyanea* + dinitrophenol. △- - -△ *Cyanea.*

However, recent observations suggest to us that the activation of a lytic enzyme probably forms only the initial link in a chain of enzymatic processes. The release of histamine and the degranulation of mast cells can also be blocked by dinitrophenol, thyroxine, cyanide, and other metabolic inhibitors.[6,14,15] FIGURE 7 illustrates the inhibitory effect of dinitrophenol (DNP) on histamine release by *Cyanea*. DNP is supposed to block intracellular oxidative energy transport by preventing the formation and enhancing the breakdown of ATP. Glucose counteracts the inhibitory action of DNP, possibly by inducing glycolytic formation of energy-rich phosphates (FIGURE 7). The energy dependence of the histamine-release process is also indicated by the fact that the presence of glucose in the incubation fluid enhances the histamine release.

Aside from indications that the energy required for histamine release from mast cells can be delivered by aerobic or anaerobic carbohydrate metabolism, nothing is known about the nature of the energy transport behind the release process. Where in this process the energy is required can be at present only a matter of speculation. It might be used for functions such as transporting the histamine-carrying granules out through the cell membrane or for the passage of the histamine-releasing agent into the mast cells.

We have not studied the action of the *Cyanea* principle on human mast cells, but it seems reasonable to assume that the skin symptoms produced by contact with *Cyanea* tentacles are due to the action of the histamine liberator introduced into the skin. This liberator then produces release of histamine from the mast cells in the area of contact. Of course, other substances, too, will be released from the degranulated mast cells. Mast cells from some animal species contain serotonin and perhaps other active amines. We have shown, in Stockholm, that another substance appears when mast cells are degranulated: a "slow-reacting substance," SRS.[1] This SRS produces gradual contraction of smooth muscle, for example, of guinea pig ileum. We have found that SRS probably forms in the mast cell when the cell undergoes degranulation and histamine is released.[13] The substance is probably a phospholipid rest. What role SRS plays in histamine release from the mast cells is thus far unknown.

Histamine liberators presumably are not uncommon in nature. In Stockholm we have extracted a partly purified, fairly active histamine-liberating principle from eelworm (*Ascaris*) of hog and horse.[2,3,15] Similar principles probably occur also in shellfish, strawberries, and other agents known to cause allergic reactions in hypersensitive individuals.

The reader may be able to provide useful suggestions on marine organisms likely to contain histamine-liberating principles. The agent that we extract from *Cyanea* is not, I suppose, very toxic, although when it is more highly purified it may well turn out to be a fairly active substance. We have not yet tried to purify it to the same degree as we have with a similar principle from *Ascaris*. The *Ascaris* factor obtained from hogs releases histamine from mast cells in concentrations below 1 μg./ml. In these concentrations the *Ascaris* extract causes a distressing itch, redness, and local edema in human skin. However, for various reasons it would be of great interest to us to find a material of high toxicity from which we could extract and purify more easily an active histamine-liberating substance. I believe that by studying the chemistry of histamine liberators occurring in nature, we may learn much about the mechanism by which antigen-antibody reactions—or should I say allergic reactions—cause histamine release. Judging by our observations in Stockholm, antigens release histamine by activating the same enzymatic process in the mast cells as that activated by the natural histamine liberators.

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ANTIBODYLIKE MATERIALS OF MARINE INVERTEBRATES*

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Animals whose habitat is the marine environment are faced with problems of resistance and immunity similar to terrestrial creatures. Agents infectious to marine organisms have produced epizootics, and a variety of toxins and venoms are described in this monograph. Aside from these deleterious agents, which are so dramatic in their effect, there are more subtle interactions between marine forms. The description of associations between particular marine organisms is a constant element in descriptive ecology. Our knowledge of the immune mechanisms employed by marine animals in dealing with the deleterious effect of other organisms in the environment is extremely limited. In particular, the production of antibody by invertebrates in general has remained a matter of uncertainty. Where activity suggestive of antibody has been observed, it has usually been of a nonspecific kind.

Most investigations have attempted to detect precipitation of soluble antigens or the agglutination of particulate ones. These two antigen antibody reactions may be expressed as follows:

$$\mathrm{SAn} + \mathrm{Ab} \rightleftharpoons \underset{\text{complex}}{\mathrm{SAn\text{-}Ab}} \rightharpoonup \text{precipitate},$$

$$\mathrm{PAn} + \mathrm{Ab} \rightleftharpoons \underset{\text{complex}}{\mathrm{PAn\text{-}Ab}} \rightharpoonup \text{agglutinate},$$

where SAn = soluble antigen, PAn = particulate antigen, and Ab = antibody. This approach to the problem is made difficult because it demands a prior knowledge of the conditions required for such visual demonstrations of antibody. Where precipitation or agglutination has been observed, interpretation of the observation is not easy. Clotting of fluids or other nonspecific effects on the physical state of the antigen may be the cause of the positive result.

I have employed a somewhat different approach. My method of detection depends on the antibody's ability to combine with its antigen regardless of visual manifestation. Materials from immunized invertebrates are added to measured amounts of soluble antigen and calibrated rabbit antiserum. The possible effects of invertebrate antibody may be illustrated by the following equations:

$$\mathrm{SAn} + \mathrm{IAb} + \mathrm{RAb} \rightleftharpoons \mathrm{SAn\text{-}RAb} \rightharpoonup \begin{array}{l}\text{expected amount of precipitate;}\\ \text{indicates no IAb}\end{array} \tag{1}$$

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$$\underset{?}{\rightleftharpoons} \begin{matrix}\text{SAn-IAb}\\ + \\ \text{SAn-RAb}\end{matrix} \rightarrow \text{less precipitate; indicates blocking IAb} \tag{2}$$

$$\underset{?}{\rightleftharpoons} \text{IAb-SAn-RAb} \rightarrow \text{more precipitate; indicates non-blocking IAb} \tag{3}$$

$$\underset{?}{\rightleftharpoons} \begin{matrix}\text{IAb-SAn}\\ + \\ \text{RAb-SAn}\end{matrix} \rightarrow \text{more precipitate; indicates precipitating IAb} \tag{4}$$

where SAn = soluble antigen, IAb = invertebrate antibody, and RAb = rabbit precipitating antibody. Reactions **3** and **4** may be differentiated by testing IAb with SAn alone. The course of the precipitin reaction is followed by repeated measurement of the optical density of the mixture in a Beckman DU Spectrophotometer at 340 mμ. Temperature is maintained at 25° C. The effect of the invertebrate material on the rabbit precipitating system is observed. Deviations from the expected course of precipitation by the rabbit Ab suggest a combination between invertebrate antibody and its antigen, and the final yield of precipitate, as determined through the use of the usual procedures of the quantitative precipitin technique, provides a check on any deviations observed.

Certain criteria may be imposed in assessing any activity observed. The activity must be inducible; that is, a correlation between the appearance of activity and the immunizing injection must be observed. The activity must not be associated with the degradative effect on the antigen that might be produced by an inducible enzyme. Finally, the activity must show a degree of specificity compatible with that of the antibody.

The intertidal anemone *Anthopleura elegantissima* is the invertebrate that Benjamin Yardley and I have most studied. Our preliminary observations, which we have submitted for publication, consisted of the following. Animals were injected with 1 mg. of Bovine Serum Albumin, Fraction V (BSA).* At 2-day intervals groups of 5 anemones were individually extracted with artificial sea water. The addition of these extracts to the rabbit precipitating system produced a deviation from both the expected course of precipitation and the expected final amount of precipitate.

Early extracts added protein to the precipitate and increased the rate of its formation. Later extracts inhibited the course of the reaction and decreased the amount of precipitate. The dilution of inhibitory extracts led to an addition of protein to the precipitate. It was concluded that at low concentrations the anemone antibody can be added to the rabbit antibody-BSA complex, but that at higher concentrations it is inhibitory.

The course of disappearance of the injected BSA was followed and found to be due to internal breakdown rather than excretion. The measured level of residual BSA present in extracts did not change over a period of several weeks. Therefore there is no indication that the antibodylike activity is due to enzyme activity. Specificity was found to be of a high degree, since extracts from animals injected with BSA had no effect on a rabbit precipitating system specific for human plasma albumin (HuPA).* In the corresponding experiment, the use of extracts from animals injected with HuPA did not affect the BSA system. Finally the course of synthesis and all other aspects described above were repeated with extracts obtained from small pieces of tissue removed at daily intervals from one animal.

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* Produced by Armour Laboratories, Kankakee, Ill.

These preliminary observations indicate that the activity observed satisfies the criteria mentioned above, and at least in these respects the material resembles antibody. Thus far it has not been possible to produce precipitation of antigen by this material.

May I report at this time on certain aspects of the reaction of anemone antibody at low concentrations; that is, concentrations at which the rabbit precipitating system is not markedly inhibited and the anemone antibody produces its additive effect.

TABLE 1
MIXTURES OBSERVED FOR PARTICULATION IN BECKMAN DU SPECTROPHOTOMETER

Mixture	Anemone extract	Antigen	Rabbit anti-serum	Artificial sea water
1			0.5 ml.	2.5 ml.
2		0.5 ml.	0.5 ml.	2.0 ml.
3	1.0 ml.		0.5 ml.	1.5 ml.
4	1.0 ml.	0.5 ml.	0.5 ml.	1.0 ml.

Experimental

Anemones were injected with two 1-mg. doses of BSA or HuPA at weekly intervals. Twenty days after the last injection, the animals were macerated in a Waring Blendor with 70 per cent ethanol that had been chilled to −5° C. The particulate material was collected by centrifugation at 2000 rpm in an International refrigerated centrifuge. The ethanol extraction was repeated until the supernatant was all but completely free of pigments. The sediment was next repeatedly washed with chilled distilled water. Finally the sediment was extracted with chilled artificial sea water containing 0.01 per cent merthiolate and stored at −5° C.

TABLE 1 identifies the mixtures placed in the cuvettes of the spectrophotometer. At intervals the machine was zeroed against mixture 1 and the optical densities of mixtures 2, 3, and 4 were determined. Mixture 2 contains the rabbit precipitating system alone. The level of antigen used places the system in extreme antibody excess. Mixture 3 contains the extract plus rabbit antibody and serves as a control for mixture 4, which contains the rabbit system plus the extract. The optical density in mixture 3 is subtracted from the optical density in mixture 4. Twenty days after injection there is no detectable residual antigen in the anemone extracts; however, on standing, the extract tends to darken, which makes this correction necessary.

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* Produced by Cutter Laboratories, Berkeley, Calif.

Results

A plot of time versus the optical density of the rabbit precipitating system and the corrected anemone system is shown in FIGURE 1. It may be observed that the deviation produced by the anemone antibody is not constant throughout the course of the reaction. Only deviations greater than ±0.002 can be considered significant, as this is the level of replication obtained with the rabbit system alone. Tests of the effect produced by the anemone extract when tested over the range of concentrations from 0.70 mg. to 0.07 mg. extract protein revealed a remarkable constancy in the pattern of these deviations.

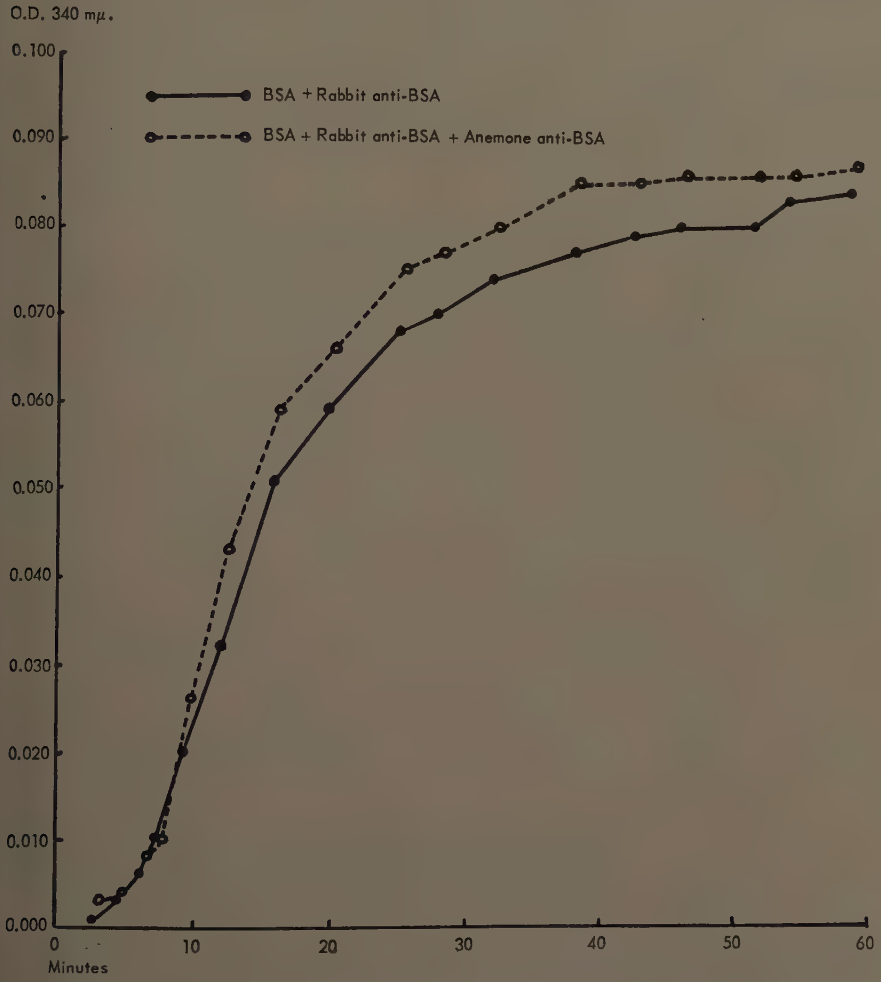

FIGURE 1. Course of particulation in the presence and absence of anemone extract.

FIGURE 2 represents a three-dimensional plot of time and concentration of extract versus deviation. Only two concentrations of extract are shown for

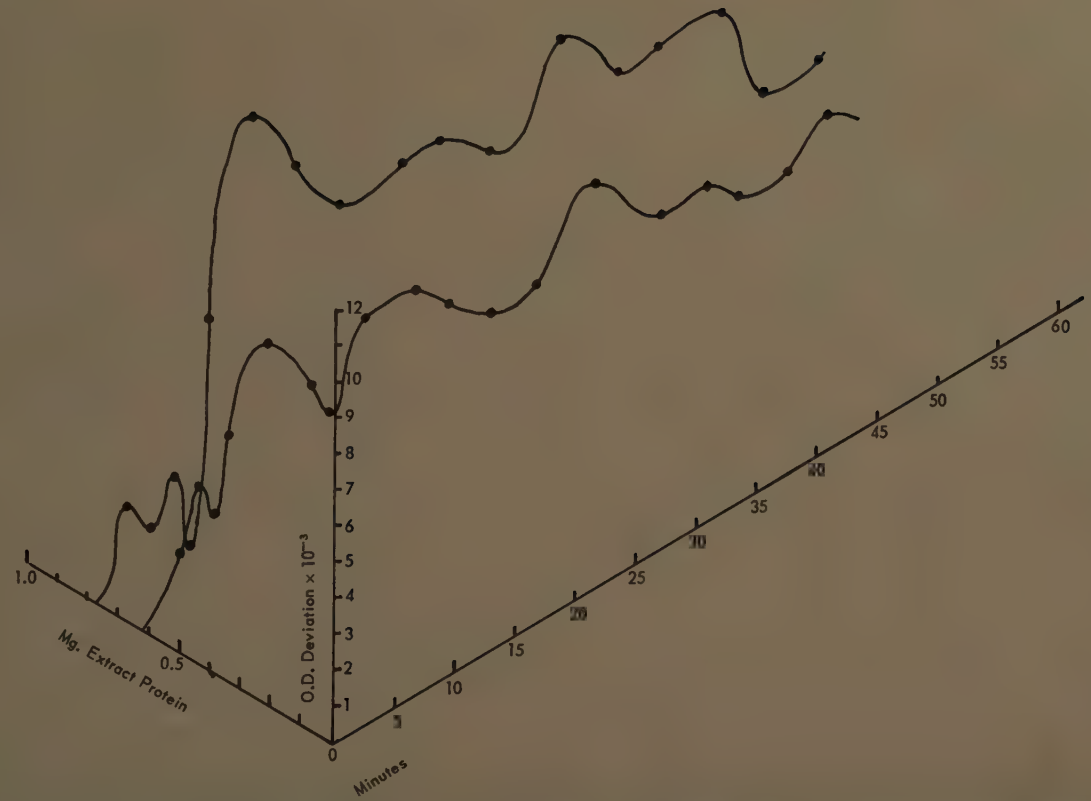

FIGURE 2. The relationship between time, concentration, and deviation from the expected course of particulation. Pooled 20th-day extract from 10 anemones that received two 1-mg. doses of BSA one week apart.

clarity; however, the essential features are maintained throughout the range. Particular peaks of positive deviation increase in height, as the concentration of anemone antibody is varied, and the series of peaks is shifted in time.

When readings are made at intervals of approximately one minute, the pattern of deviations becomes somewhat more complicated, as is revealed in the top curve of FIGURE 3.

The various prominent peaks of positive deviation have been designated by letters. The curve represents the actual points obtained from a test using 0.7 mg. of extract protein plus a composite of many curves that allows the definition of deviations less than ±0.002. Portions of the curve shown in broken line represent features not completely revealed by this particular test with 0.7 mg. of extract protein.

This observed constancy in the deviation pattern requires an explanation. As I have mentioned, the concentration of the anemone antibody governs the placement of the series of peaks in time and governs the height of individual peaks. Particulation of the antigen-antibody complex with resulting turbidity is being measured. Since the observations of Ramon[1] and Dean and Webb,[2] it has been known that the time of particulation of antigen-antibody complexes is related to the concentration of the reactants, so we can relate part of the effect to previously observed properties of antigens and antibodies. The series of peaks of deviation is less easily explained. It would suggest a heterogeneity with respect to the reactants. BSA Fraction V contains 3 to 5 per cent globulin as its main contaminant. Armour's Bovine Gamma Globulin Fraction II (BGG) was added to the system at a high concentration in an attempt to inhibit any globulin-antiglobulin reactions by excess antigen. The lower curve of FIGURE 3 shows the result. While the essential features of the deviation curve are the same, some portions have been shifted forward or backward in time. The peaks preceding E, F, and G occur earlier; those following these same reference peaks occur later in time. There has been no obliteration of peaks except for those occurring so rapidly that measurement in this test was impossible. Because of these shifts it would appear that the peaks may represent separate antigen-antibody reactions; however, none can be traced to gamma globulin acting as an antigen.

FIGURE 4 shows the deviation curves obtained when Armour's crystalline BSA is used as an antigen (top curve) and when BGG Fraction II is used at a concentration corresponding to the expected level of globulin in BSA Fraction V (middle curve). The usual set of deviation peaks may be observed with the crystalline BSA. The use of BGG at this low level does not produce a significant deviation pattern. When tested at the higher level used for inhibition by antigen, BGG does give significant deviations, but these lie beyond the M peak in time. At the bottom of FIGURE 4 is the scatter of small deviations observed when anemone anti-BSA is tested in the HuPA precipitating system. All points lie within the ±0.002 deviation limits, and there is no constancy to the pattern.

At the top of FIGURE 5 the pattern obtained with anemone anti-HuPA in the homologous system is shown. It may be seen that quite a different pattern of deviations is obtained. The results of the use of this same extract with the

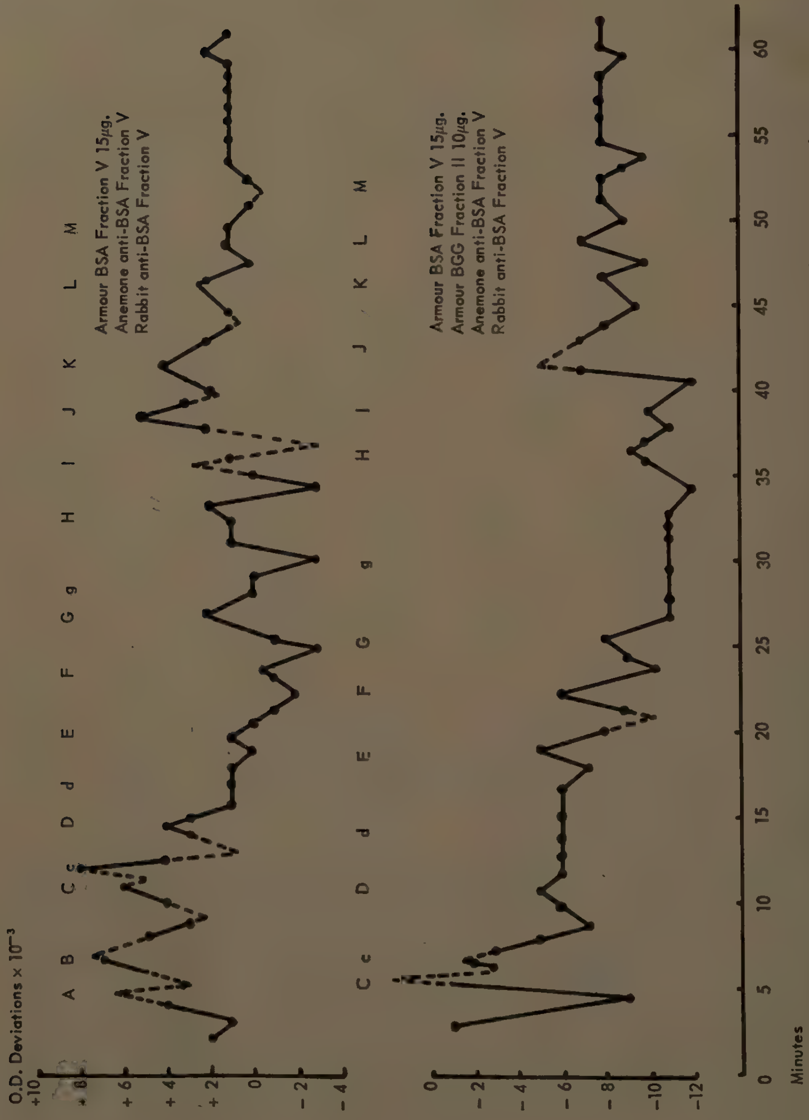

FIGURE 3. Deviations from expected course of particulation observed using various preparations.

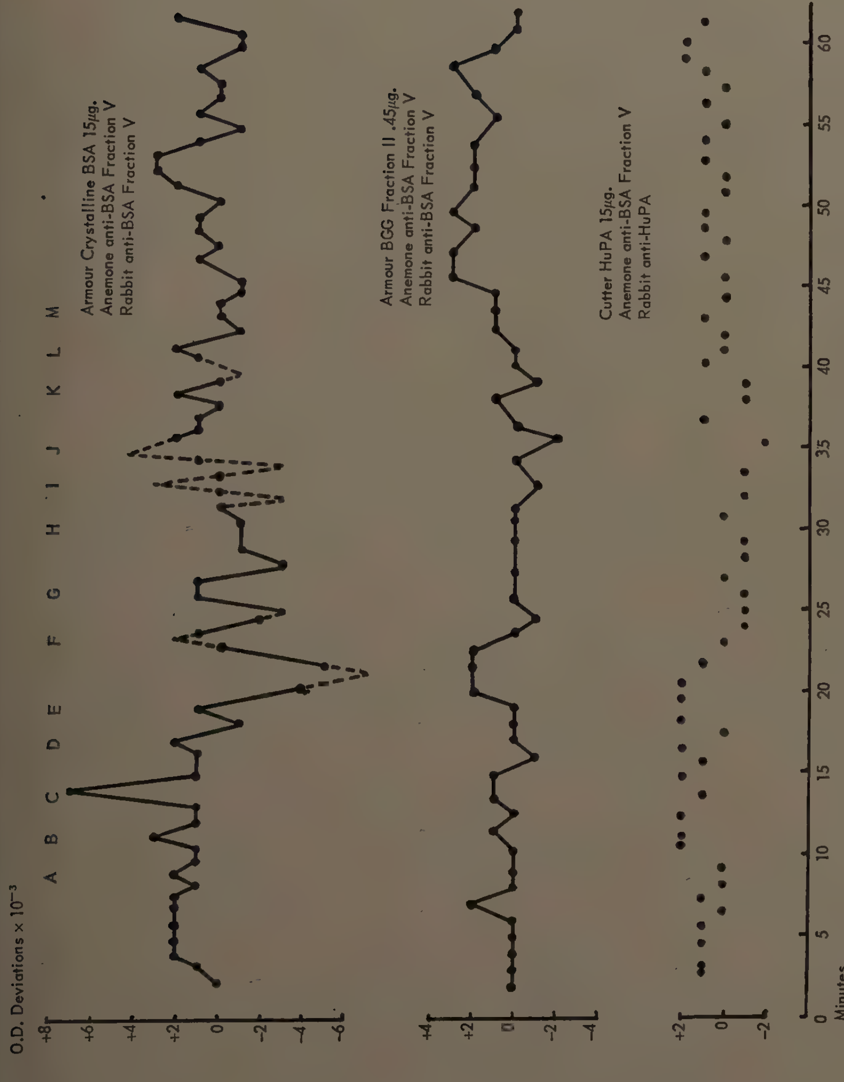

FIGURE 4. Deviations from expected course of particulation observed using various preparations.

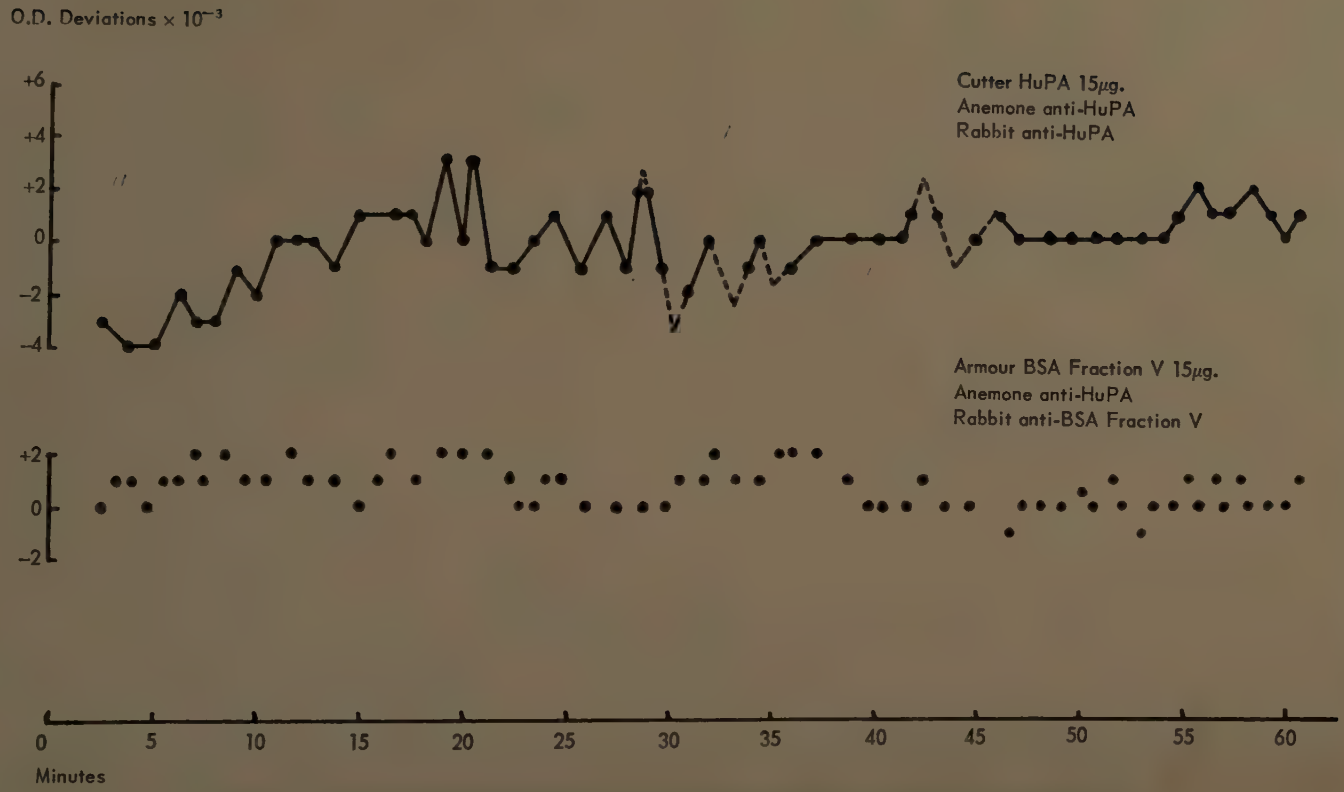

FIGURE 5. Deviations from expected course of particulation observed using various preparations.

heterologous BSA system is shown in the lower half of this figure. Again there is no significance to the deviations observed.

My findings may be summarized as follows. A highly specific material resembling nonprecipitating antibody can be produced by at least one coelenterate. Preliminary observations indicate a similar material may be produced by a mollusc, *Tegula funehralis*, and a sipunculid, *Phascolosoma agassizi*. At high concentrations, this anemone antibody inhibits its homologous rabbit precipitating system. The rabbit-blocking anti-BSA studied by Feinberg[3] shows a similar inhibitory effect in extreme excess. At lower concentrations the anemone antibody adds protein to the rabbit antibody-antigen complex. When the course of particulation in this addition phase is followed, a consistent sequence of what appear to be separate additions and inhibitions is observed. The entire pattern can be shifted in time by varying the concentration of the anemone antibody. Individual parts of the sequence can be shifted in time by varying the composition of the mixture of kinds of molecules acting as antigen.

Aside from the biological interest in this substance from the anemone, it may be of immunochemical interest as well. The detection of a kind of heterogeneity not generally observed with antigen and antibody may be indicated.

The analysis of heterogeneity by the techniques of gel diffusion rely on differences in the rate of diffusion of the antigen molecules and the inhibition of precipitation by excess antigen. The study of the course of particulation as influenced by nonprecipitating antibody may provide another procedure of a similar kind since the time of particulation of individual complexes is known to be dependent upon the concentration of the reactants.

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OBSERVATIONS ON CIGUATERA-TYPE TOXIN IN FISH*

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This is an interim report on the progress of an investigation of ciguatera in the tropical Pacific that is being carried on at the Hawaii Marine Laboratory of the University of Hawaii. For almost two years these studies have been supported not only through grants‡ but also through informal cooperation of the government of the United States Trust Territories, the United States Air Force, the medical services of the United States Army and Navy, and the Royal Air Force of Great Britain. This widespread support of the investigation reflects the gravity of the problem of poisonous fishes in the Pacific.

These investigations have been limited primarily to the toxin from the flesh of the large red snapper, *Lutjanus bohar* (Forskål), from the Line Islands, south of the Hawaiian archipelago. All of the later studies dealing with bioassay and chemical extraction have been confined to this species from Palmyra Island in the Line Islands. This restriction was imposed to insure that only one type of toxin was being investigated. While it is unlikely that other fishes causing ciguatera have toxins differing from those of *L. bohar* from Palmyra, this could not be assumed. *Lutjanus bohar* was selected as the fish for study because it is reported to be one of the most consistently toxic fish throughout the Pacific. It is generally abundant and easily caught, and it is large enough to provide adequate material for sampling. Palmyra Island was selected for logistic reasons and because of the high toxicity of the fishes there.

This report covers the work that has been done on the definition of the problem, the development of a bioassay, the initial attempts at extraction and purification of the toxin, and a preliminary inquiry into the relationship of ciguatera to toxin from a common alga.

Further Definition of Ciguatera

There is no need for a review of the occurrence or the symptomology of fish poisoning, nor for a general discussion of its possible origin, for this has been done adequately in the literature (Hiyama, 1943; Halstead, 1959; and Randall, 1958). It will suffice here to state that the fish of the tropical Pacific with flesh toxic to humans have been divided into five major categories: sharks, eels, scombroid fishes, puffers, and the various fishes bearing ciguatera toxin. Only two of these categories have been adequately studied: the puffers, wherein the chemistry and pharmacology of the toxin have been explored (*inter alios* by Yudkin, 1944; and Tsuda *et al.*, 1960); and the scombroid fishes, wherein the causa-

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tive organism, a bacterium, has been isolated and identified (Kawabata *et al.*, 1956).

One or possibly two more types of fish toxicity have come to light in the course of this investigation. The first has been named "hallucinatory mullet poisoning" (Helfrich and Banner, 1960); it has been reported from various mullet (Mugilidae) and surmullet (Mullidae) and possibly other fish from restricted areas on the reefs of Hawaii. It may also occur at Norfolk Island in the South Pacific and in the waters of South Africa. These fish, when eaten, produce a syndrome that includes hallucinations if the patient is awake or nightmares if asleep; other symptoms, which may include itching of the mouth and throat and locomotory difficulty, are variable. The disease is distinguished from ciguatera by the rapid onset of and recovery from the symptoms, by the hallucinations, and by the lack of gastrointestinal involvement. It was established on the basis of case histories; adequate medical studies have not been made on it nor has it yet been produced in the laboratory with either test animals or human subjects.

The second type, which tentatively may be called clupeoid poisoning, appears to be widespread through the islands of the more tropical Pacific and Indian Oceans, for vague reports on it (chiefly in personal correspondence) have originated in the Marshalls, Societies, New Caledonia, Fiji, Indonesia, and Ceylon. The fish implicated is a sardinelike fish (possibly *Sardinella*) which becomes toxic only during certain seasons, as in Fiji during the *balolo*-worm season (November and December); at other times the fish may be eaten with impunity. During the toxic season it may be lethal, as in the case of the deaths of five members of a Fijian village caused by eating a sardinelike fish in December, 1955 (*Pacific Islands Monthly*, Jan., 1956). No medical details of the symptoms are available, and little else is known about the occurrence of these toxic fish. However, if they are seasonally toxic, they appear to be markedly different from fish bearing ciguatera that are consistently toxic throughout the year.

Some previous observations on ciguatera have been confirmed through personal interviews, through correspondence, and through laboratory tests. These observations include the regionality of ciguatera and the changes in the pattern of toxicity over the years.

Ciguatera has long been reported to be of regional occurrence, limited to definite islands and, possibly, to narrow regions of those islands. This type of distribution has been confirmed in the Line Islands. At Palmyra we have found by mongoose-feeding tests (see below) 95 per cent of a total of 61 specimens of *L. bohar* over 50 cm. long (taken near the channel entrance) to be highly toxic. At Christmas Island, 357 miles distant, 51 per cent of a total of 65 specimens of *L. bohar* of similar size caught to the north of the entrance to the lagoon were toxic. At Fanning Island, between Palmyra and Christmas Islands, the fish were highly toxic in 1946, but the toxicity has decreased since that time (Ross, 1947; Halstead, 1959). At Washington Island, near Fanning Island, M. N. Fagg, resident manager of the coconut plantation, wrote in a personal communication in 1959, that "there are no fish known to be poisonous at Washington. . . ." He estimates that the native population of 310 Gilbertese consume perhaps 400 to 500 lb. of fish per day, of which *Takananingo* (Gilbertese for *L. bohar*) is "eaten in quantity."

An indication of the very narrow regionality may be found in the data relating to fish caught north and south of the lagoon entrance at Christmas Island. About one half of the large fish caught to the north were toxic, yet only one of the five caught to the south of the entrance, about six miles distant, was toxic. This difference in toxicity was reported also by the Gilbertese residing upon the island.

Other authors have cited the changing toxicity over the years in specific island areas (Randall, 1958; Bartsch *et al.*, 1959; Halstead, 1959). A new and dramatic change in toxicity of fish occurred in late 1959 at Midway Island in the Hawaiian chain. Only one previous attack of fish poisoning had been known to occur there, but in 1944 about 24 persons were poisoned by eating a "black sea bass" (Lee and Pang, 1945*a*, 1945*b*); thereafter the eating of "colored" fish was discouraged by the United States Naval authorities. However the inhabitants, military and civilian, ate small "papio," *Caranx cheilio* Snyder, caught in abundance in the lagoon. This fish was esteemed and contributed regularly to the diet of many of the residents; one woman who was interviewed stated that she had eaten the fish on the average of once a week for two years. In the latter part of October, 1959, the species suddenly became toxic and in the following two months more than 25 individuals were treated for the characteristic ciguatera symptoms of varying intensity at the Naval Hospital. The present toxicity of the fish is not known, for the residents have ceased to eat this species.

TABLE 1

SPECIES OF FISH FOUND TO BE TOXIC BY MONGOOSE-FEEDING TESTS*

Species	Common name	Locations
Lutjanus bohar (Forskål)	Snapper	Marquesas Is., Line Is., Marshall Is.
Lutjanus gibbus (Forskål)	Snapper	Line Is.
Lutjanus monostigmus (Cuvier and Valenciennes)	Snapper	Society Is.
Lethrinus miniatus (Forster) Bloch and Schneider	Snapper	Marshall Is., Line Is.
Lethrinus variegatus Cuvier and Valenciennes	Snapper	Line Is.
Monotaxis grandoculis (Forskål)	Snapper	Line Is.
Plectopomus leopardus (Lacepède)	Grouper	Marshall Is.
Epinephelus fuscoguttatus (Forskål)	Grouper	Line Is.
Cephalopholis argus Bloch and Schneider	Grouper	Line Is., Society Is.
Variola louti (Forskål)	Grouper	Line Is.
Acanthurus triostegus triostegus (Linnaeus)	Surgeonfish	Line Is.
Ctenochaetus striatus (Quoy and Gaimard)	Surgeonfish	Line Is.
Caranx ignobilis (Forskål)	Jack	Marquesas Is., Line Is.
Caranx lugubris Poey	Jack	Marquesas Is.
Gymnothorax javanicus (Bleeker)	Moray Eel	Line Is.

* Identification after Schultz *et al.* (1953). Fishes of the Marshall and Mariana Islands. Bull. U. S. Nat. Mus. **1** (202).

It was not the purpose of this phase of the investigation to study the toxicity of fish other than *L. bohar*. However, incidental to the main investigation, certain fish were tested by the mongoose-feeding tests and were found to be toxic. The results are presented in TABLE 1.

All of the species cited in this table caused similar response in the test animals and the reaction varied only with the toxicity of the sample. This is notable because moray eels have been suggested to have a type of toxin distinct from that of ciguatera, yet specimens of *Gymnothorax javanicus* (Bleeker) from Palmyra caused symptoms that could not be distinguished from those caused by the other fish. This would indicate that the toxin in moray eels may be the same as that of ciguatera (see discussion of Randall, 1958), but until chemical studies are completed no positive statement can be made.

Development of a Bioassay

The studies on a bioassay may be divided into three phases: a search for a reliable quantitative feeding test, the development of a test by injection of an extract of the fish flesh, and an investigation of the reliability of a previously published test.

Based on the bioassay developed for testing *Gonyaulax* toxin in shellfish, a general test had been developed for poisonous fish that consisted of an intraperitoneal injection of an extract into mice (Ishihara, 1918; Yasukawa, cited in Hiyama, 1943; Macht & Spencer, 1941; Hiyama, 1943). The extract was either an alcoholic or an aqueous extract of the flesh or viscera. This test was used on all types of toxic fish encountered and was standardized by Halstead and his co-workers (Halstead & Bunker, 1954) who first used acidified water and later used distilled water as the extraction medium.

As the reactions in humans result from the ingestion of fish, the primary test used in this investigation was based on the reactions of animals fed potentially toxic fish.

Initially, feeding experiments were carried on with cats; however, because of legal restrictions in Hawaii, the use of cats as test animals is difficult. It was found that the mongoose, *Herpestes mungo*, an animal introduced to Hawaii, reacted with symptoms similar to those of the cat. The chief difference is that cats, when fed poisonous fish, will often regurgitate the test meal, thereby making it difficult to estimate the amount of toxic fish retained; mongooses, on the other hand, always retain all of the toxic meal. They can be fed up to 15 per cent of their body weight of raw fish directly, or they will eat dried fish or extracts that are mixed with raw eggs.

The reactions of the mongooses are also similar in some respects to those of the humans. These reactions may be classified in five stages, based on the maximal response in the mongoose within 48 hours after a single test feeding: 0, no reaction; 1, slight weakness and flexion of the forelimbs; 2, slight motor ataxia, more pronounced flexion of the forelimbs, and weakness of the hind limbs; 3, moderate motor ataxia with weakness and partial paralysis of limbs and body musculature; 4, acute motor ataxia and extreme weakness, capable of only limited movement, or coma; and 5, death.

All reactions are accompanied by a tarry, loosely formed stool; reactions 3, 4, and 5 (before death) are sometimes accompanied by excessive salivation and trembling.

While the symptoms of the disease, especially of the more acute cases, are neurological, no morphological changes in the nervous system were observed in post-mortem examinations. Post-mortem examinations of 58 mongooses killed in feeding tests revealed that the only pathological condition was a tendency to develop gastric ulcers. When this was studied further, no apparent relationship was found between the ulcers and the toxic meals; the ulcers may be the result of the encagement of wild mongooses.

Two mongooses were fed sublethal doses of toxic fish for a month; the fish used had been found to be lethal when fed in a single feeding at 10 per cent of body weight. The test mongooses were fed intermittently 1 per cent of body weight per day as required to maintain symptoms. They soon developed an arched posture, moved with difficulty, and showed other symptoms of intoxication. They also became extremely sluggish and irritable. When the symptoms became too acute, the test animals were returned temporarily to a diet of nontoxic fish. One mongoose was fed a total of 107.7 gm. (22.6 per cent body weight) of toxic fish over a 32-day period; the other was fed 81.9 gm. (20.2 per cent of body weight) over a 33-day period. At the end of the experiment, the two test animals and two controls were killed, preserved in formalin with the central nervous system exposed, and were examined by Asao Hirano, a neuropathologist with the National Institute of Neurological Diseases and Blindness Research Station at the Guam Memorial Hospital, Mariana Islands. Hirano could find no pathological change in the central nervous system. Another pathologist of the same institution, R. Wilhoite, examined other tissues and observed evidence of hepatotoxic change; however, he requested more material to confirm his observations.

Mongooses, however, are not completely satisfactory as test animals, for they come from a wild population and are biologically variable; moreover, they consume too much fish to permit the replicate testing necessary to establish an LD_{50}. A series of invertebrates and vertebrates, selected on the basis of general availability, were tested for susceptibility to the toxin. Carnivores or omnivores, such as crabs, snails, and rats, were fed fresh or dried fish directly; filter feeders or detrital feeders, such as *Paramoecium* or *Artemia*, were fed on finely-ground fish suspended in the culture; and herbivores, such as fruit flies and pigeons, were fed on finely ground fish mixed with their normal food or were force-fed. The results are presented in TABLE 2.

There is no assurance that all animals listed in TABLE 2, assuming they are susceptible to the toxin, ingested sufficient quantities of toxic fish to cause a reaction. This is especially true in those cases where the fish was suspended in the culture medium, but the difficulty of feeding them otherwise ruled them out as satisfactory test animals. Also questionable are the results of some tests where the animals, such as parakeets or mice, would avoid eating fish or fish-contaminated food. However it is notable that a number of animals have existed without ill effects from a week to several months on a diet of fish that was lethally toxic to mongooses; they include such animals as a fish (*Acanthurus xanthopterus*), the lobster and crab (*Panulirus* and *Scylla*), and the laboratory rat.

Only three of the animals tested, other than the cat and mongoose, developed recognizable symptoms. These were the white mouse, the crayfish, and the turtle. With these, other difficulties were experienced. The mouse did not eat raw fish. When dried fish was compressed into a pellet with Purina Laboratory Chow, the mouse would eat only part and scatter much more of it, thus rendering the mouse unsuitable for quantitative assay. The crayfish reacted in the same general manner as the mongoose; it would lose its ability to right itself, develop locomotory ataxia, and eventually die; however, as was the case with the mouse, it was difficult to estimate the amount ingested. The turtle was even more unsatisfactory, for its sole reaction was to withdraw within its shell; it never ate enough to cause death.

As a quantitative bioassay based on the mongoose-feeding test was not practicable for reasons cited above, and as no other animals were found that would lend themselves to a bioassay, the mouse injection test was re-examined. The principal prerequisite of this test is the extractibility of the toxin from flesh with water. This was feasible with tetrodotoxin, which is initially a water-soluble toxin, but studies by other workers (Hashimoto, 1956; McFarren and Bartsch, 1959) and at our laboratory (Banner and Boroughs, 1958) indicated that the toxin causing ciguatera could not be initially extracted with water.

A number of workers have indicated, furthermore, that the injection of aqueous extracts into mice could not be relied upon. Among these are: Hashimoto (*loc. cit.*), who worked with *Sphyraena picuda* Bloch & Schneider; McFarren and Bartsch (*loc. cit.*), who worked with various species including *Lutjanus gibbus* (Forskål) and *Lethrinus miniatus* (Forster); Goe and Halstead (1955), who reported the intoxication of a human by fish that had been shown to be nontoxic by the test; Brock and Takata (unpublished results), who worked with *L. bohar* from Palmyra; and Banner and Boroughs (*loc. cit.*), who also worked with *L. bohar*. Thus if the mouse injection test were to be used, it would have to be carefully re-examined.

During the early phases of this work it could be shown by mongoose-feeding tests that all toxin could be removed from the flesh with 90 per cent to 100 per cent ethanol. This extract, after removal of ethanol, was injected intraperitoneally into mice. While the extract would kill mice, a similar extract of a nontoxic fish, either a tuna or a Hawaiian lutjanid (*Pristipomoides microlepis* [Bleeker]) would also cause death at 1.5 to 2.0 times the lethal level of the toxic extract.

However, a more highly purified extract gave satisfactory differentiation between the toxic fish and the control. In the preparation of the six highly toxic specimens of *L. bohar* listed in TABLE 3 an average of 131 gm. of dried, ground flesh was extracted with 400 ml. of 95 per cent ethanol in a Soxhlet extractor for 24 hours; the extract was concentrated to 50 ml. The concentrate was then diluted with 150 ml. of distilled water, yielding approximately 25 per cent alcoholic solution. The resulting solution was washed with three 100-ml. portions of petroleum ether; it was discovered by feeding tests that the aqueous solution carried the toxin and that the petroleum ether extract was nontoxic. The aqueous alcoholic solution was next extracted with 300 ml. of diethyl ether in three portions, which removed the bulk of the toxin. This ethereal extract was evaporated to dryness, yielding an average of 96.7 mg. (738 mg./kg. of dried fish flesh) of residue containing the toxin.

This partially purified extract, as well as the discarded fractions, were investigated by intraperitoneal injection in a peanut oil vehicle in laboratory mice

TABLE 2
SUMMARY OF ANIMALS TESTED WITH TOXIC *LUTJANUS BOHAR*

Phylum/Class	Animal	Common names	Method of feeding*	Reaction
Protozoa/Sarcodina	*Amoeba proteus*		Suspension in culture of raw and dried flesh; v.f.	None
/Ciliata	*Paramoecium caudatum*		Same	None
Coelenterata/Anthozoa	*Fungia scutaria*	Mushroom coral	Pieces of raw flesh; v.f.	None
Platyhelminthes/Turbellaria	*Planaria* sp.		Same	None
Aschelminthes/Rotifera	(Various genera)		Suspension in culture of dried flesh; v.f.	None
Arthropoda/Crustacea	*Artemia salina*	Brine shrimp	Same	None
	Daphnia sp.	Water flea	Same	None
	Atya bisulcata	Stream shrimp	Pieces of raw flesh; v.f., f.f.	None
	Leander debilis	Marine shrimp	Same, v.f.	None
	Macrobrachium sp.	Stream shrimp	Same	None
	Procambarus clarki	Crayfish	Same	Paralysis to death
	Panulirus japonicus	Spiny lobster	Same	None
	Paribaccus antarticus	Slipper lobster	Same	None
	Portunus sanguinolentus	Swimming crab	Same	None
	Scylla serrata	"Samoan" crab	Same	None
	Pseudosquilla ciliata	Mantis shrimp	Same	None
/Insecta	*Periplaneta* sp.	Cockroach	Pieces of dried flesh; v.f.	None
	Dermestidae	Dermestid beetle	Same	None
	Musca sp.	House fly	Pieces of raw flesh; v.f.	None
	Calliphoridae	Blow fly	Pieces of raw flesh, fresh to putrescent; v.f.	None
	Drosophila sp.	Vinegar or fruit fly	Alcoholic extract mixed in culture medium; v.f.	None
Mollusca/Gastropoda	*Achtina fulica*	African land snail	Pieces of raw flesh; v.f.	Questionable
/Pelecypoda		Various clams	Suspension of ground flesh into incurrent syphon	Expulsion of suspension
Echinodermata/Echinoidea	*Tripneustes gratilla*	Sea urchin	Raw flesh; v.f.	None
Chordata/Osteoichthyes	*Lebistes reticulatus*	Guppy	Pieces of dried flesh; v.f.	None
	Xiphophorus helleri	Swordtail	Same	None
	Pranesus insularum	Silversides	Pieces of raw flesh; v.f.	None
	Acanthurus xanthopterus	Surgeon fish	Same	None

TABLE 2—(*Continued*)

Amphibia	*Bufo marinus*	Toad	Pieces of raw flesh; f.f.	None
Reptilia	*Pseudomys scripta elegans*	Gecko	Same	None
	Leticlodactylus lugibris	Painted turtle	Pieces of raw flesh; v.f.	General withdrawal
Aves		Parakeet	Alcoholic extract evaporated on food; v.f.	None
		Pigeon	Pieces of raw flesh; f.f.	Regurgitation
		Chicks	Ground dried flesh; v.f.	None
Mammalia		Rabbit	Pieces of raw flesh; f.f.	None
		Guinea pig	Same	None
		Laboratory rat	Pieces of raw and dried flesh; v.f.	None
		Laboratory mouse	Pieces of raw flesh; v.f., f.f.	Variable reaction

* v.f., voluntary feeding; f.f., force feeding.

(closed colony Carworth Farms Webster strain). Mice weighing between 19 and 21 gm. were used. It was found that the petroleum ether extract and the aqueous alcoholic residue from both toxic and nontoxic fish would cause death in mice when injected at a level of 3.0 to 5.0 mg./gm. of mouse weight. However, the ethyl ether extract from toxic fish would produce kills at the level of 0.2 mg./gm., while the ether extract of the control (*P. microlepis*) caused no symptoms at 1.5 mg./gm., or seven times the lethal level of the toxic fish.

TABLE 3
EVALUATION OF BIOASSAY METHODS FOR TOXIN FOUND IN *LUTJANUS BOHAR*
Preparation of Extracts, Testing Methods, and Classification of Response as Discussed in the Text

Specimen	Mongoose-feeding test Response vs. per cent body weight				Intraperitoneal injection in mice						
					Water extract (5 mice used for each specimen)	Diethyl ether extract, in mg. extract/gm. mouse. Figures represent mean time to death in minutes of two mice tested at each level					
	5	10	10	15		0.1	0.3	0.5	0.7	1.0	1.5
Highly toxic											
1	2	3	4	3	N.D.[*]	(348)[†]	189	130	63	—[‡]	—
2	4	5	4	5	N.D.	(1627)	249	155	111	69	89
3	5	5	4	5	N.D.	N.D.	240	81	90	43	30
4	2	3	4	5	N.D.	(360)	90	44	63	62	43
5	2	3	4	4	N.D.	N.D.	261	139	78	72	49
6	5	3	5	4	N.D.	308	128	101	89	—	—
Moderately toxic											
7	—	—	1	1	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	—
8	2	1	2	2	N.D.	N.D.	788	850	108	—	—
9	—	—	1	2	N.D.	N.D.	(2800)	750	—	124	—
10	—	—	1	2	N.D.	N.D.	—	N.D.	—	(367)	—
11	—	—	0	1	N.D.	N.D.	N.D.	1252	—	1044	—
12	—	—	1	0	N.D.	N.D.	N.D.	(1620)	—	—	—
Nontoxic											
13	—	—	—	0	N.D.	N.D.	N.D.	N.D.	—	2800	—
14	—	—	—	0	N.D.	N.D.	N.D.	N.D.	—	N.D.	—
15	—	—	—	0	N.D.	—	N.D.	N.D.	N.D.	N.D.	N.D.
16	—	—	—	0	N.D.	—	N.D.	N.D.	N.D.	N.D.	1000
17	—	—	—	0	N.D.	—	N.D.	N.D.	N.D.	N.D.	—
Pristipomoides microlepis (control)	0	0	0	0	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.

[*] N.D. signifies no deaths in the 72-hour test period.

[†] Figures in parentheses are times to death in one mouse of pair tested, the other living through test period.

[‡] Dash indicates no tests made at this level.

The symptoms produced in the mouse by injection were quite different from those found in the mongoose after ingestion. The mice would develop immediate diarrhea and later would become inactive and display excessive salivation and lachrymation, but they would show no paralysis; immediately prior to death the mice would display convulsive spasms for a few moments. On gross autopsy the only observable disorder was a pulmonary congestion. This difference in reaction raised the question whether the same toxin was being tested by the two methods.

Therefore, mice were fed toxic extracts, and mongooses were given intraperitoneal injections of the extract. Whether the mice were fed dried toxic fish mixed with their food or toxic extracts mixed with their food they would die showing symptoms similar to those elicited by injection; to achieve a response equal to that obtained by injection required a dosage that varied from at least equal to twice the injected amount. When the extract was mixed with egg and fed to mongooses, or when it was injected into mongooses, they would die with the same response that they would exhibit after eating raw toxic fish. The difference in symptoms was thus demonstrated to be a difference in reaction of two species to the same toxin.

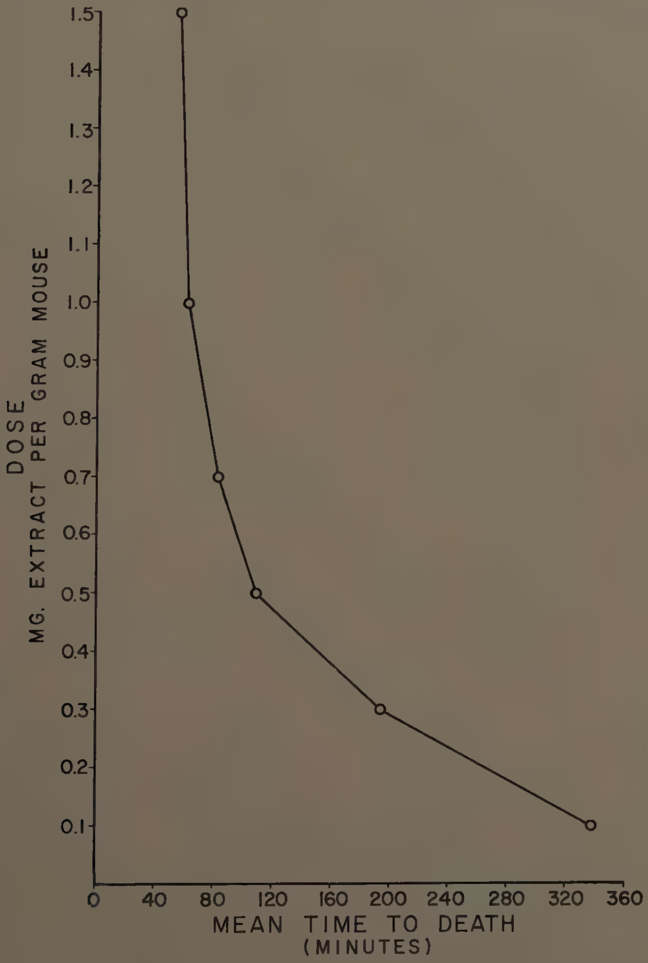

FIGURE 1. Mean time to death in mice injected at six dose levels with diethyl ether extracts of six highly toxic *L. bohar*, as shown in TABLE 3.

In order to determine the effects of size of mice on the reactions to a standard dosage, mice ranging in size from about 10 gm. to 30 gm. in five weight groups were injected with standard dosages of a mixture of highly toxic fish at a level of 1 mg./gm. of mouse. The time to death is given in TABLE 4, and the averages show that both the smallest and the largest mice are less sensitive and more variable in their reaction than are those in the 20 to 25 gm. range. The variability in time to death is given in TABLE 5.

TABLE 4

RESPONSE OF MICE OF VARYING SIZES TO STANDARD TOXIC DOSAGE IN WATER AND OIL VEHICLES

All mice injected intraperitoneally with 1 mg./gm. mouse of standard toxic mixture

Oil vehicle				Water vehicle			
Mouse weight (gm.)	Average weight (gm.)	Time to death (min.)	Av. time to death (min.)	Mouse weight (gm.)	Average weight (gm.)	Time to death (min.)	Av. time to death (min.)
9.0	9.6	66	79.7	10.1	9.4	—	103.3*
9.6		63		8.8		58.5	
9.5		97		10.1		166.5	
10.2		67.5		8.4		87.5	
9.6		105		9.5		101	
14.3	14.2	59.5	72.6*	14	14.9	48	57.0*
14.6		60.5		15.1		—	
14.1		—		14.8		50	
14.1		59.5		15.5		62.5	
13.8		111		15		67.5	
20.4	21.6	72	66.8	20	19.7	55.5	55.6*
18.9		65		19.6		61	
19.7		64		20		51	
20.3		65		19		—	
19.5		68		20		55	
23.7	24.0	62	61.6	25	24.8	60	51.3
23.8		68		25		43	
23.4		59.5		25		57	
24.0		59		25		51.5	
24.9		59.5		24		45	
32.2	31.1	71	78.8	32.9	32.9	68	66.3
31.5		74.5		32		70	
29.6		91		33.8		61	

* In some cases one mouse did not die within the test period of 3 hours; in these cases the average is based on the 4 mice that died.

At the same time that the reaction of mice of various weights was investigated, the response of mice to the toxin in a water vehicle instead of an oil vehicle was explored. The thick oil resulting from the evaporation of the diethyl ether extract was mechanically stirred with water until a stable emulsion was achieved. This emulsion, when injected intraperitoneally, produced a weight-response curve similar to that obtained with an oil vehicle, but indicated a slightly shorter time to death in all except the 10-gm. group.

The reliability of the mouse injection test was further explored in conjunction with the water-extraction test and with the mongoose-feeding test. Three groups of five specimens of *L. bohar* from Palmyra were used, representing highly toxic, moderately toxic, and nontoxic fish, as established by mongoose-feeding tests. To determine toxicities each fish was individually fed to a number of mongooses. In the highly toxic category, one mongoose was fed at 5 per cent body weight, two at 10 per cent body weight, and one at 15 per cent body weight; if at least one animal displayed a grade 4 response at 10 per cent body weight feeding, the fish was classified as highly toxic. For moderately toxic fish, the criterion was no greater than a grade 2 response at a 15 per cent body weight feeding. Because the available nontoxic *L. bohar* were much smaller in size, only a single feeding at 15 per cent body weight was possible; this produced no symptoms.

The tests based on injection of a water extract of the flesh were carried out by a modification of the published procedure of Halstead and Bunker (1954).

TABLE 5

VARIABILITY IN TIME TO DEATH OF THE MICE GIVEN IN TABLE 4

Sample size	Vehicle	Av. weight (gm.)	Mean time to death (min.)	Standard deviation of time to death (in sample)
5	Oil	9.6	79.7	19.72
4	Water	9.4	103.3	45.66
4	Oil	14.2	72.6	25.58
4	Water	14.9	57.0	9.50
5	Oil	21.6	66.8	2.88
4	Water	19.7	55.6	4.24
5	Oil	24.0	60.4	3.70
5	Water	24.8	51.3	7.36
3	Oil	31.1	78.8	10.68
3	Water	32.9	63.0	4.73

Two ml. of distilled water and 1 gm. of fish flesh were homogenized in a blender for 2 min.; this slurry was then centrifuged at 2000 rpm (900 g) for 25 min. One milliliter of the clear supernatant fluid was injected intraperitoneally into each of four mice weighing 15 to 25 gm. Reactions were observed for 26 hours and placed into four groups: immediate death; immediate symptoms and delayed death; immediate symptoms and recovery; no symptoms.

The following changes were made in the Halstead and Bunker technique: (1) 25 gm. of fish flesh were used in all cases; (2) the container of the blender was cooled to assure temperatures below 40° C., which prevented excessive precipitation of proteins; and (3) the resulting slurry was first centrifuged for 5 min., then decanted and recentrifuged as specified by Halstead and Bunker.

The diethyl ether extracts were prepared as outlined above; all were injected intraperitoneally in a peanut oil vehicle.

In addition to the three groups of *L. bohar*, a control was run with *Pristipomoides microlepis* from the Honolulu fish market.

The results are presented in TABLE 3; the accompanying graph (FIGURE 1) shows the curve of the mean time to death of the mice injected with varying amounts of the highly toxic group shown in the table.

The mongoose-feeding test and the injection tests of water and diethyl ether extracts were tried on a specimen of *Caranx ignobilis* (Forskål) that had caused mild ciguatera in a human. This jack (or pompano or ulua) weighed 38 lb., and was caught in the lagoon entrance at Palmyra. A large portion, possibly 2 lb., was baked and eaten by an individual who later developed a characteristic itch of the mouth and skin, a sensitivity in the hands to heat and cold, and some "difficulty" in the leg muscles that manifested itself by pain and fatigue, especially when climbing stairs. Other members of the victim's family who ate smaller portions noticed no symptoms. The uncooked portions, saved in a frozen condition, were fed to mongooses at 5, 10, and 15 per cent body weights; there was no reaction at 5 per cent, marginal reaction (grade 1 response) at 10 per cent, and slight reaction (grade 2 response) in two mongooses at 15 per cent. There was no reaction to the injection of the water extract in five mice. The diethyl ether extract was injected in an oil vehicle at 0.1, 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 mg. of extract per gm. of mouse. No deaths resulted from the lower three injection levels; 2 of 4 mice died at 1.5 mg./gm., and all died at the three higher levels; in the three uppermost injection levels the average times to death were 98, 36.5, and 34.5 min., respectively.

While the systematic LD_{50} was not determined for adequate numbers of mice to establish a truly quantitative response for the diethyl ether extract, the following conclusions may be drawn:

(1) Feeding tests with mongooses may be used for qualitative determination of toxicity in *L. bohar*, but there is too much variation in the animals to permit reliance on the reactions of one or two mongooses to establish the degree of toxicity.

(2) The published technique of Halstead and Bunker (*loc. cit.*) of injection of a water extract of the flesh of *L. bohar*, and probably also of *C. ignobilis*, gives no indication of the toxicity of the fish. If other types of fish that have been characterized as causing ciguatera have the same toxin as *L. bohar*, it is most unlikely that this test will give a reliable indication of their toxicity.

(3) The injection of a partially purified diethyl ether extract, as outlined above, presents the most reliable method by which to test for toxicity in *L. bohar*. This method is likely to produce quantitative results once the assay is standardized with large numbers of test fish and test animals and is based on LD_{50} data.

Initial Attempts at Isolation of Toxin

At this time it is not possible to report fully on the isolation of the toxin. However, a brief review of the initial isolation and purification studies of the toxin will be presented.

First, a possible decomposition or loss of the toxin in the course of air-drying the flesh at 90° C. was explored. Replicate mongoose-feeding tests with raw and dried *L. bohar* indicated that there was no loss of toxicity; this confirmed an earlier finding by Banner and Boroughs (1958).

Second, the extractability of the toxin by various solvents was investigated. Thirty gm. of a uniform mixture of dried toxic *L. bohar* were extracted with different solvents, usually with two successive portions of 60 ml. each. The solvent was evaporated to dryness and the residue weighed. The combined extraction residues and the extracted fish flesh, after complete removal of solvent, were fed to mongooses separately. The results are shown in TABLE 6.

TABLE 6 shows, *inter alia*, that ethyl alcohol was the extraction solvent of choice. In order to determine the most efficient method of alcohol extraction of the maximum amount of toxin from the flesh, three methods were explored. In the first, 100 gm. of dried flesh were extracted with hot ethanol in a blender with three portions of 200 ml. each for 5 min.; after solvent removal the combined extracts produced 4.27 gm. of extractable matter. Then the residual fish flesh was re-extracted with ethanol in a Soxhlet for 16 hours, which removed an additional 7.79 gm. of extract. Mouse injection tests showed that both blender and Soxhlet extracts contained toxin.

TABLE 6

EFFICIENCY OF VARIOUS SOLVENTS IN EXTRACTING TOXIN FROM DRIED FLESH OF *LUTJANUS BOHAR*, AS INDICATED BY MONGOOSE-FEEDING TESTS

Solvent (reagent grade)	Extracted (gm.)		Reaction to	
	1st Extraction	2nd Extraction	Extract	Residue
Water	4.35	*	None	2, 3
Ethanol, 90%	1.15	1.06	3	None
Ethanol, 100%	1.29	0.45	5	None
Methanol	1.52	0.94	None	2
Butanol	0.74	0.23	None	†
Acetone	0.25	0.10	2	None
Chloroform	0.30	0.13	2, 3	None
Diethyl ether	0.27	0.01	3, 4	None
Benzene	0.27	0.04	None	None
Petroleum ether (B.P. 30–60° C.)	0.21	0.02	None	3

* Single extraction only.
† Not tested.

Third, Bligh and Dyer's (1959) triple solvent extraction method was tried, but it was found that this method also failed to extract much of the toxin that could subsequently be removed by Soxhlet extraction with alcohol. Therefore the best method found to date to remove the toxin is by Soxhlet extraction.

After the elaborate purification method, outlined under the section on bioassay, had been developed, an abbreviated method was tried, wherein the dried flesh was extracted directly with diethyl ether, thus bypassing the extraction with alcohol and the washing with petroleum ether. Here it was found that the diethyl ether extract of the toxic fish was not as toxic in terms of mg./gm. lethal dose in mice as that obtained by the elaborate method, and furthermore that the nontoxic control fish (*P. microlepis*) would kill mice at nearly the same dose levels.

Further purification of the diethyl ether fraction cannot be reported upon at this time, but it may be stated that through the use of column chromatography with solvents of varying polarity, a noncrystalline but possibly homogenous extract has been isolated. The yield of this extract is 32 mg./kg. of wet flesh extracted from highly toxic fish; this extract, when injected into mice at 65 γ/gm., will kill in 14 min.

Relation of Ciguatera to Toxin from Lyngbya majuscula

Various authors (Dawson *et al.*, 1955; Randall, 1958; Dawson, 1959) have suggested that the toxin of ciguatera is passed through the food chain to the large carnivores, and that its ultimate source may be a blue-green alga. It has further been suggested that the source may be algae of the genus *Lyngbya*, a widespread form known to be toxic.

During the summers of 1958 and 1959 there were outbreaks in Hawaii of a "swimmer's itch" produced by strands of *Lyngbya majuscula* Gomont that had become entangled in close-fitting swimmer's trunks. During the summer of 1959, workers at the Hawaii Marine Laboratory investigated the spread of dermatitis-producing *L. majuscula* (Banner, 1959), which presented a public health problem. At the same time the *Lyngbya* toxin was compared, in a preliminary way, with the toxin that produced ciguatera from *L. bohar*.

L. majuscula would produce severe and persistent blisters and later lesions of the skin when it was applied in a patch test for 6 to 18 hours (FIGURE 2). The same alga when dried and mixed with mouse food would kill test mice that ate the food. When an alcoholic extract was made and injected intradermally in guinea pigs and rabbits, it would cause erythema and vesicular eruptions; the alcoholic extract when evaporated on mouse food would kill mice.

Not all strains of *L. majuscula* about the island of Oahu were toxic. For example, the alga from Laie and Kailua, two beaches on Oahu about 22 miles apart, was toxic during both summers, but the same species from Kaneohe Bay, located between Laie and Kailua, proved to be consistently nontoxic by skin tests.

Possible relationship of the toxins from *Lyngbya majuscula* and from *L. bohar* was investigated in three ways:

(1) *Solubilities.* While great caution must be exercised when one compares the solubility of a given component that may be present in two mixtures of widely differing composition, nevertheless the toxins from the fish and the alga appear to have different solubility characteristics. The toxin from both the alga and the fish could be extracted with 90 and 100 per cent ethanol, but the toxin from the alga could also be extracted with hot water or cold petroleum ether, although not as efficiently as with alcohol. The toxin from *L. bohar*, on the other hand, could not be extracted with either of these solvents. When the ethanol extract from the alga was purified in a manner parallel to that used for *L. bohar*, as outlined previously, some of the toxin appeared in the petroleum ether washing, as well as in the diethyl ether fraction.

(2) *Patch tests.* Alcoholic extracts of *Lyngbya* would produce erythema and blisters when applied as patch tests to human subjects; similar extracts of *L. bohar* caused no reaction.

(3) *Geographic spread of both conditions.* *Lyngbya* dermatitis has been known in Hawaii by scientific observation for at least two years, and much longer by

accounts of fishermen. However, in Hawaii only two cases of fish poisoning of the ciguatera type have definitely been established. In both cases the fish was not caught near known areas of toxic *Lyngbya.* The toxic seaweed areas, on the other hand, are heavily fished for many species of fish. Moreover at Palmyra, where the percentage of fish bearing ciguatera is very high, none of the *L. majuscula* tested produced any reaction in skin tests. It is worthy of note that Habekost *et al.* (1955) found algae from Palmyra (including *L. majuscula*) to be toxic by their tests using intraperitoneal injection of purified aqueous extracts of the algae.

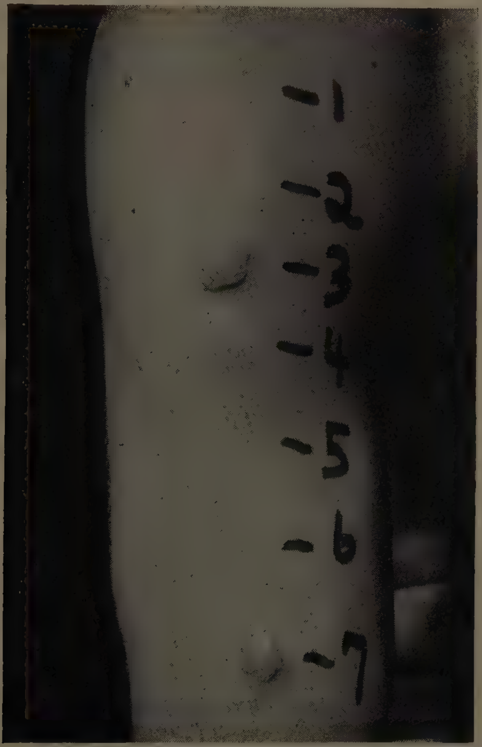

FIGURE 2. Reaction in a 6-hour patch test to *Lyngbya majuscula* Gomont. Upper arm of subject showing the results of seven samples of *L. majuscula* collected from swimming beaches about the island of Oahu, Hawaii. Note that in addition to the conspicuous blister from samples 3 and 7, there is a mild reaction to sample 4.

It appears, therefore, from these initial considerations that the compound or compounds from some strains of *Lyngbya majuscula*, which cause dermatitis, are chemically not identical with the toxin that produces ciguatera from the ingestion of *L. bohar.*

Summary

(1) Ciguatera should be distinguished from hallucinogenic mullet poisoning and possibily from a yet-undescribed condition resulting from eating seasonally toxic fish.

(2) The narrow regionality of fish causing ciguatera and the changing patterns of toxicity in the central Pacific have been further confirmed.

(3) Fifteen species of fish causing similar reactions in feeding tests are listed; the validity of the separation of *Gymnothorax* poisoning from ciguatera is questioned.

(4) Two methods of bioassay for ciguatera-producing fish are presented, based on *Lutjanus bohar*, a red snapper from Palmyra Atoll. The first is a feeding test using mongooses as test animals; the second, which shows good potential to become a quantitative test, is based on the intraperitoneal injection into standard mice of an alcoholic extract of fish flesh, subsequently purified by washing with petroleum ether and re-extraction with diethyl ether.

(5) More than 37 species of invertebrates and vertebrates were tried for possible use in feeding tests for bioassay; of these only three, a crayfish, a turtle, and a laboratory mouse, reacted.

(6) A previously used test, which has been the criterion of toxicity in numerous toxic fish surveys, was found to be unreliable for the fish used in this study.

(7) A procedure is given for the initial extraction and purification of the toxin from fish flesh. Discussion of eventual isolation of the pure toxin has been reserved for further experimental confirmation.

(8) The appearance of a strain of *Lyngbya majuscula* that produces dermatitis in humans upon contact is discussed, together with its possible relationship to ciguatera from fish; it was concluded that there is at present no evidence that the toxins from the alga and from the fish are identical or even similar.

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MARINE BIOTOXINS. I. CIGUATERA POISON: SOME BIOLOGICAL AND CHEMICAL ASPECTS*

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Ciguatera is a neurotoxic form of ichthyosarcotoxism caused by the ingestion of a poison occasionally found in marine fishes. The disease is largely confined within a broad circumglobal belt indicated by the shaded area in FIGURE 1. This belt extends approximately from latitude 35° N. to 34° S., and only in rare instances has ciguatera been suspected of occurring beyond these boundaries. The disease is most prevalent in the tropical Pacific and the West Indies. Ciguatera poison is not confined to any particular species of marine fish, but is known to occur in a wide range of unrelated species. Toxicity among ciguatera-producing fishes fluctuates widely from one specimen to the next. A member of one species may be highly toxic in one region and nontoxic in another. Ciguatera poison has been found in fishes taken at every season of the year. However there is some evidence that certain species, such as *Sphyraena barracuda*, the great barracuda, are more toxic during the reproductive season than at other times.

The origin of ciguatera poison has been discussed by several scores of investigators. Most writers adhere to the theory that the poison is contracted through the feeding habits of the fish, but there is no uniformity of opinion as to specifically what agent or organism is primarily responsible. The caustive agents suggested include manchineel berries, *Cocculus* berries and other poisonous shoreline terrestrial plants, marine algae, protozoa, sponges, jellyfish, corals, sea anemones, molluscs, annelid worms, crustaceans, starfishes, sea cucumbers, fishes, inorganic chemical agents, climatic changes, parasitic infections, normal and pathological metabolic functions in the fish, and even such an unlikely source as moonlight. There is evidence to show, however, that ciguatera is in no way related to any known form of bacterial food poisoning. Preliminary stomach content, toxicological, and algological analyses have placed certain species of blue-green marine algae under strong suspicion as possible sources of ciguatera poison.[1,2,3]

The purpose of the present paper is to define the disease and to point up certain aspects of the problem. This is the first of a series of reports that will be concerned with the biology of the disease and the chemical and pharmacological characteristics of the poison.

Problems of primary concern at this time relate to the origin of the poison and its transmission to other organisms; the relationship of incidence of toxicity in fish to the size, sex, age, feeding habits, and reproductive cycle of the fish; relationships of toxic phytochemical constituents in marine algae to ciguatera;

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and chemical and pharmacological characteristics of the poison. Answers to these fundamental problems must be found if intelligent methods of controlling and treating the disease are to be developed.

Historical Background

The earliest reference to ciguatera, published in 1555, is generally credited to Peter Martyr, the first historian of the West Indies.[4] The Spanish explorer Quiros, in his visit to the New Hebrides in 1606,[5] had his entire crew poisoned from eating "pargo." In 1675 Locke referred to numerous poisonous fishes occurring in the Bahama Islands.[6] Anderson in 1776[7] reported an incident in which Captain James Cook's crew was poisoned at Malekula, New Hebrides, as a result of eating a "red pargo," probably one of the species of *Lutjanus*.

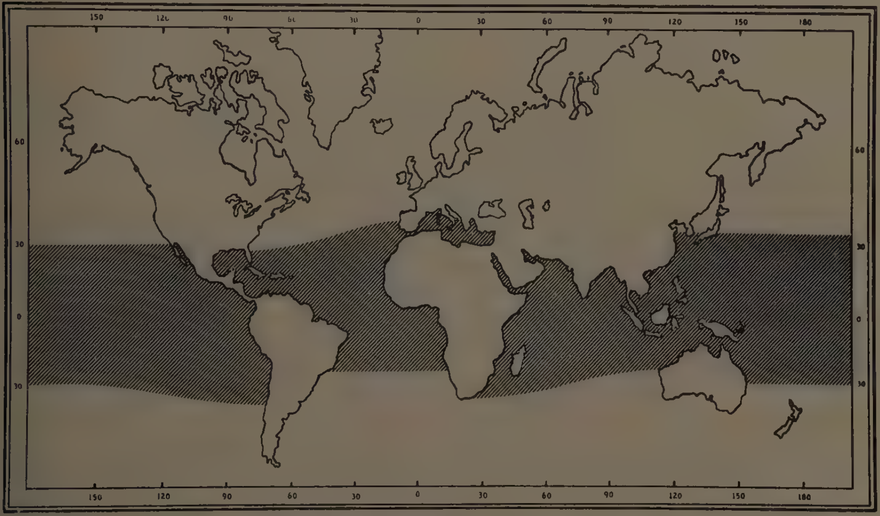

FIGURE 1. The circumglobal belt (shaded area) in which ciguatera intoxications have been known to occur.

Lipp, in his doctoral thesis of 1829,[8] reported on more than 60 species of poisonous fishes of the West Indies and discussed the symptoms that these fish produced. Steinbach's paper of 1895[9] was probably the first publication on the poisonous fishes of the Marshall Islands. Becke[10] (1901) estimated that up to 50 per cent of the fishes in some parts of the Marshall Islands were poisonous to eat, and Matsuo,[11] after conducting a survey of the poisonous fishes in the vicinity of Jaluit Island, reported that 20 per cent of the reef fishes were toxic. A similar survey was conducted at Saipan by Yasukawa.[12] Gilman, in 1942, published a review of the problem of poisonous fishes in the Virgin Islands[13] and, in this same year, Hiyama,[14] under the sponsorship of the Japanese Imperial Navy, conducted and reported on one of the most extensive field studies ever undertaken on the poisonous fishes of Micronesia. In this same vein, Whitley prepared for the Allied Forces of World War II a manual identifying the poisonous fishes to be found in the vicinity of the Great Barrier Reef.[36]

Clinical reports on ciguatera have been published by Vonfraenkel and Krick,[15] Lee and Pang,[16] Schneck,[17] Cohen *et al.*,[18] Ross,[19] Halstead and Lively,[20] Halstead,[21] and others.

Starting in 1945, the results of a series of surveys on the distribution of poisonous fishes in the tropical Pacific have been reported by Halstead and his associates.[22-26]

There is also available an annotated bibliography prepared in 1955 by Whitley and Halstead[27] on the poisonous fishes of Australia.

In 1956 Hashimoto published the first paper defining some of the elementary physical and chemical characteristics of ciguatera poison.[28] Little work in this area has been done since that time, and the structure of the toxin still remains unknown.

One of the most critical epidemiological reports that has appeared to date is by Bartsch *et al.*,[29] based on their studies in the Marshall Islands.

Reviews of the over-all problem of ciguatera have been published by Gudger[30] and Randall.[31]

Despite the fact that much has been written about ciguatera, little of factual nature is known regarding the origin of the poison or its chemical and pharmacological properties. There is an ever-increasing amount of evidence indicating that ciguatera-producing fishes are more widespread than was formerly believed, and that the disease appears to be on the increase in certain portions of the Indo-Pacific region. The problem presents a serious threat to the further economic development of the fishery resources of the tropical Pacific. Moreover, ciguatera poison, as a substance with intriguing chemical and pharmacological properties, introduces a challenging research problem.

Biological Assay Methods

Finding a suitable method of testing for the presence of ciguatera poison has been a problem to which much effort has been directed in the past. Various techniques have been employed. Most of these methods have been reviewed.[22,30,32] The mouse intraperitoneal injection test and feedings to kittens have proved to be of the greatest utility. More recently Banner and Boroughs have developed an oral feeding test using the mongoose.[33] Mongooses, however, are difficult to handle and are not generally available as a laboratory test animal in most parts of the country. None of the biological assay methods employed to date has been completely satisfactory. The mouse intraperitoneal test, widely used as a bioassay for toxic substances, has been repeatedly subjected to criticism on the basis of its seeming lack of sensitivity and reliability as an assay for ciguatera poison. The problems of false positives on one hand and insensitivity on the other have been matters of continual concern. Tests conducted on more than 12,000 fish specimens representing several hundred species of marine fishes have revealed that a majority of these specimens will not give toxic reactions. This tends to negate the criticism that the mouse will yield false positives because of sensitivity to fish protein injected intraperitoneally as an aqueous homogenate. Despite the fact that ciguatera toxin is insoluble in water, a centrifuged aqueous homogenate of poisonous fish tissue, administered parenterally to mice, is capable of causing a reaction characteristic of a ciguatera intoxication. As the concentration of poison being given is probably low it is likely that mice are quite sensitive to ciguatera toxin. In an aqueous homogenate prepared from the flesh of a fish, the poison may be found with the fat in the proteinaceous debris, it may be bound in certain lipoprotein complexes, or it may be in the form of a water-lipid emulsion. It has been demonstrated by several investigators that oral feeding tests utilizing mice or rats are insensitive.[33] In our experience the mouse intraperitoneal test, despite its recognized weaknesses, has proven to be the most satisfactory assay for use in screening large numbers of fishes.

Until recently we have employed the kitten feeding test for the detection of ciguatera poison in limited quantities of fish specimens to be used in chemical analyses. The gross symptoms in kittens are leg weakness, generally developing first in the rear legs and spreading to the front, ataxia, muscular incoordination and, in some instances, vomiting, diarrhea, hypersalivation, complete prostration and, occasionally, death.

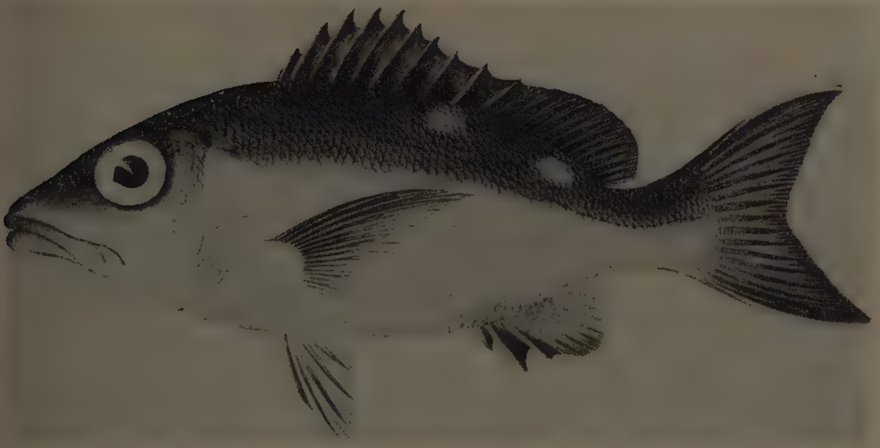

FIGURE 2. *Lutjanus bohar* (Forskål), the red snapper.

Repeated administration of toxic material apparently does not lead to immunization, sensitization, or toxin accumulation. In one experiment designed to evaluate these possibilities a male cat weighing about 2.1 kg. was fed six doses of weakly toxic fish muscle over a period of eight weeks. In general, each feeding brought a repetition of symptoms, such as mild vomiting, slight weakness in the rear legs, and some ataxia. In this experiment, as with all others carried out in our laboratory, the muscle of *Lutjanus bohar* (Forskål), red snapper (FIGURE 2), was used as the source of ciguatera toxin. The fish were taken by hook and line in about 30 feet of water in the vicinity of Sand Island, Palmyra Atoll, during October 1958.

In another experiment of slightly different design a female cat weighing about 1.4 kg. was fed six doses of strongly toxic *L. bohar* muscle over a period of six weeks. The data and results are shown in TABLE 1. In this rather limited series there was observed a response varying at least qualitatively with the dosage, and there is no evidence of either an immunization or sensitization phenomenon. One may conclude that with proper feeding intervals cats may be reused several times for testing ciguatera-producing fishes.

Cats, although quite sensitive to ciguatera poison, do possess some disadvantages. Being relatively large test animals, they require large amounts of material. Also, cats show a rather narrow tolerance between the minimum toxic dose and a dose that elicits a vomiting response. Vomiting vitiates the calculation of dosage. Finally, cats are readily susceptible to various viral infections that have an extremely high mortality rate. Unless one works with naturally hyperimmunized animals (the use of vaccines proved of little value), there is the difficulty of having disease symptoms superimposed on toxicity reactions and, in such cases, the results are difficult to interpret.

In attempting to develop an assay more suitable for small-scale chemical fractionation techniques it was found that ciguatera toxin has an inhibitory effect on the action potential of excised frog sciatic nerve. The test finally devised is conducted in the following manner. An acetone and ether soluble extract of fish muscle is emulsified into frog Ringer's solution (Ling's No. VII[34]) by means of ultrasound, and the excised nerve, mounted on an electrode assembly arranged for the detection of the action potential, is bathed in the emulsion for 55 min. At 55 min. and every 5 min. thereafter up to a total of 70 or 80 min. the action potential, generated under a stimulation voltage that is maximal for the untreated nerve, is measured by means of a low level preamplifier and a cathode ray oscilloscope. A final potential appreciably below the standard for the nerve indicates a toxic emulsion.

TABLE 1

MULTIPLE FEEDING EXPERIMENT WITH FEMALE CAT WEIGHING 1.4 KG. FED SEVERAL DOSES OF TOXIC MUSCLE OF *LUTJANUS BOHAR*

Day of feeding	Dosage (% of body weight)	Onset of first symptoms (hours)	Duration of symptoms (days)	Symptoms
1st	1.25	>3.5 < 18	∼1.5	Diarrhea, vomiting, leg weakness, ataxia
6th	1.25	9	∼1.5	Leg weakness, ataxia
13th	1.25	>6 < 22	∼2.5	Leg weakness, ataxia
20th	1.9	6	∼2	Diarrhea, vomiting, leg weakness, ataxia
27th	2.5	>7 < 22	∼5	Diarrhea, vomiting, severe leg weakness, ataxia
42nd	2.5	>5 < 21	Cat sacrificed	Severe leg weakness, ataxia

In order to determine the reliability of the frog sciatic nerve test a number of red snappers were assayed by both cat oral feedings and the frog nerve test. The cat, based on a maximum dosage of 4 per cent of body weight, indicated that 22 of a group of 55 fish were toxic. By comparison, the frog nerve showed that 20 fish were toxic if the test was conducted using an emulsion composed of 10 ml. of frog Ringer's solution and the extract from 1.5 gm. of fresh frozen fish muscle. A drop in the percentage of retention of action potential of 25 per cent or more at 70 min. was considered to indicate a toxic fish. The 2 cases in which the cat and the frog test results disagreed involved fish that were only weakly toxic to cats.

Relationship of Weight to Toxicity in Lutjanus bohar

Currently on hand for study are 190 *L. bohar* caught at Palmyra Atoll in October, 1958. All of these fish have been tested for toxicity by the frog nerve method, and 59 (31 per cent) have given a toxic reaction. The relationship that exists between size and incidence of toxicity has been revealed by dividing the fish into weight groups and calculating the percentage of toxic fish in each group. The correlation, expressed graphically in FIGURE 3 where the percentage of toxic fish is plotted against weight group, shows an initial low incidence of toxicity and then a very sharp increase starting at about 2800 gm. (Group V). Below 2800 gm. only 18 per cent of the fish are toxic; above this weight 69 per cent are toxic.

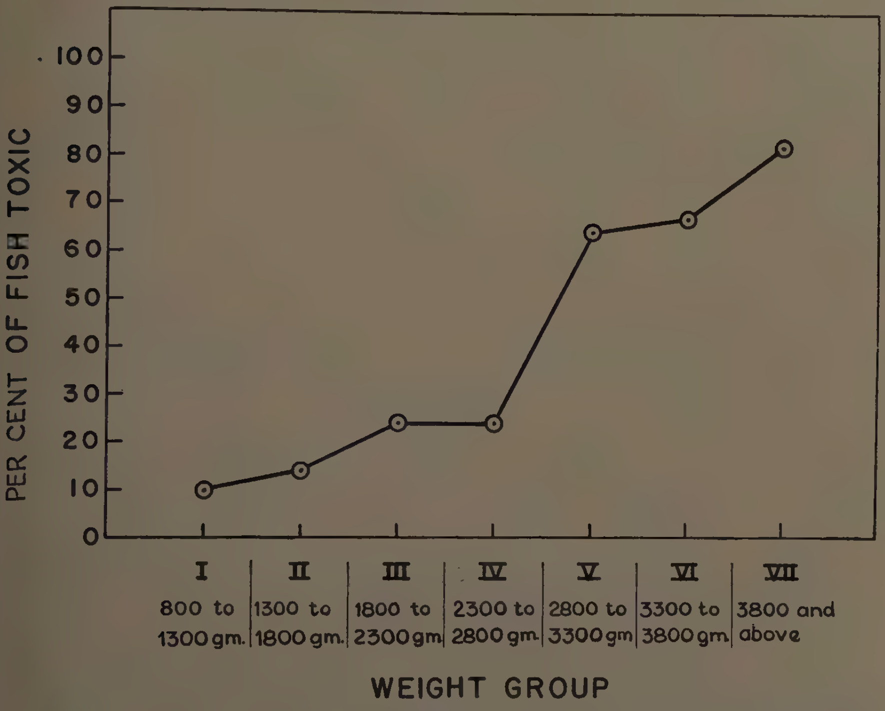

FIGURE 3. The relationship between weight and incidence of toxicity in *L. bohar* from Palmyra, Line Islands.

Besides exhibiting a higher *incidence* of toxicity, the larger red snappers also apparently have a higher *concentration* of poison. We have considered the per cent retention of action potential in the frog nerve test a rough measure of the concentration of toxin in the muscle of the fish. Arbitrary degrees of toxicity have been established as follows: (1) strong, 0 to 25 per cent retention; (2)

moderate, 25 to 50 per cent retention; (3) weak, 50 to 75 per cent retention. All toxic red snapper have been classified according to these ratings and, in addition, divided into the weight groups defined in FIGURE 3. The percentage of fish strongly, moderately, and weakly toxic in each weight group have been plotted against weight groups, with the results shown in FIGURE 4. Although Group III seems to show anomalous behavior, the trends are unmistakable. The incidence of weakly toxic fish (*dotted line*) falls off as there is an increase in the size of the fish, while the incidence of strongly toxic fish (*solid line*) behaves in a reciprocal fashion. As might be expected, moderate toxicity (*dashed line*), which represents an intermediate stage, rises to a maximum and then falls off. Although these trends are present, it should be emphasized that relatively small red snappers can be strongly toxic, and large ones can be weakly toxic or even nontoxic. How these facts, and those concerning relationships between toxicity and weight, are related to feeding habits, the reproductive cycle, or some environmental factor, is not known.

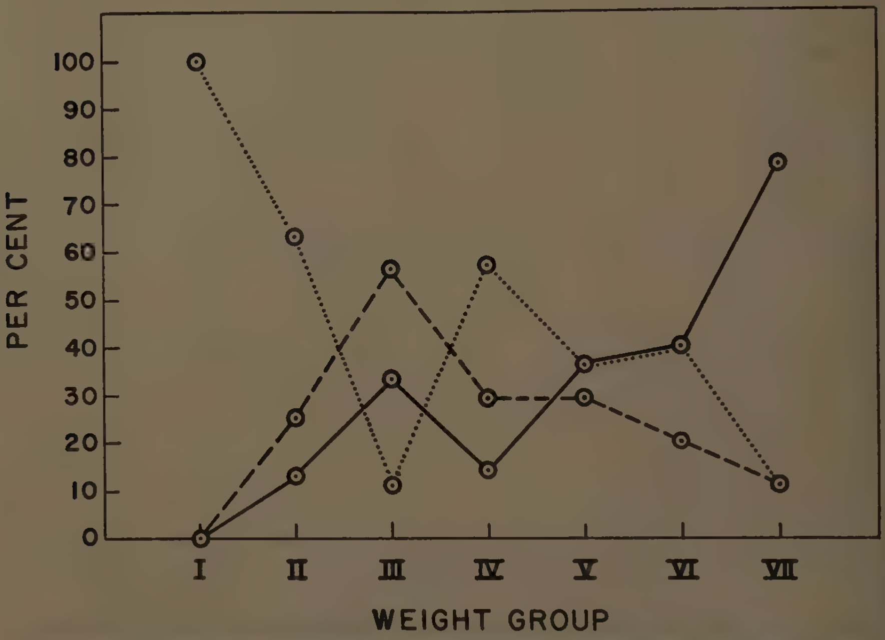

FIGURE 4. Percentage of toxic fish (*L. bohar*) that are strongly toxic (*solid line*), moderately toxic (*dashed line*), or weakly toxic (*dotted line*) plotted against weight group (as defined in FIGURE 3).

Chemistry of Ciguatera Poison

Very little work has been done to elucidate the physical and chemical characteristics of ciguatera toxin. A search of the literature reveals the following information: the toxin is heat stable;[14,33] soluble in ether, petroleum ether, chloroform, acetone, methanol, and 90 per cent ethanol;[28,33] and insoluble in water and absolute ethanol;[33] it is somewhat sensitive to oxygen;[33] and, according to Halstead and Ralls,[35] it is dialyzable.

Much of the chemical work on ciguatera toxin that has been carried out in our laboratory to date has been more or less of a preliminary nature. Regarding further solubility characteristics, it has been learned that the crude toxin is soluble in benzene, ethyl acetate, isopropyl alcohol, methylene chloride, methyl ethyl ketone, and carbon tetrachloride. Dilute solutions (5 per cent) of sodium hydroxide and hydrochloric acid failed to remove ciguatera toxin from an ether solution. The base extraction removed about 30 per cent of the original crude toxic oil dissolved in the ether, and this base-soluble material, recovered from the neutralized (sulfuric acid) sodium hydroxide solution by ether extraction, was nontoxic to a cat. An extraction by dilute hydrochloric acid subsequent to the treatment with base dissolved only about 10 per cent of the original crude toxic oil, and this acid-soluble material likewise proved to be nontoxic to a cat. Apparently the ciguatera toxin molecule contains no functional groups normally considered to be acidic or basic.

Fractionation Techniques

As the first step in the isolation of pure ciguatera toxin we have worked out a procedure for the extraction of the crude material from fish muscle. A sample of muscle, mixed with acetone, is whipped to a slurry in a high speed homogenizer (VirTis 45). After suction filtration on a Büchner funnel the filter cake is re-extracted in a similar manner and then discarded. The acetone filtrates are combined. A flocculent, fatty precipitate that sometimes forms at this point is removed and discarded. The acetone solution is evaporated to a small volume of cloudy white liquid by means of a rotary evaporator. Solvent removal is hastened by the application of a vacuum (water pump) and heat (water bath maintained at about 50° C.). The milky liquid residue is extracted with three portions of ether, the ether layers being retained and combined. The solvent is removed using a vacuum evaporation on a rotary evaporator, and the resulting oily residue is extracted with three small portions of acetone. The combined acetone extracts, after filtration, are evaporated under vacuum on a rotary evaporator to yield the crude toxin. At this stage the poison is a light yellow, mobile oil. In preparing extracts for screening large numbers of fish by the frog nerve test, only the first acetone extraction and the subsequent ether extraction are used.

In an attempt at further fractionation of the crude toxin and the possible development of a chemical test, some exploratory paper chromatographic experiments have been conducted. The following solvent systems were used: butanol-acetic acid-water (25:6:25, v/v/v), isopropyl alcohol-water-ammonium hydroxide (10:1:1 and 20:5:1, v/v/v), isopropyl alcohol-water (2:1, v/v), chloroform-methanol (4:1, v/v), and methanol-acetone (2:1, v/v). With all systems the toxin either moved with a major amount of the impurities at the solvent front or was lost. Exposure of the toxin to air and solvents was minimized by the use of circular chromatograms that were rapidly developed by the application of centrifugal force.

Further work is continuing on fractionation techniques in the hope that pure ciguatera poison may be isolated. Plans for the future include determination of additional physical and chemical properties and an attempt to elucidate the structure of the toxin molecule.

Summary

Ciguatera, a neurotoxic disease caused by a poison occasionally found in certain marine fishes, has been known since the 16th century. A voluminous literature covers various aspects of the disease, such as the origin of the poison in nature, clinical symptoms, species, and geographical distribution of ciguatera-producing fishes. The origin of the poison remains obscure and, exclusive of some meager solubility data, nothing is known about the toxin's physical, chemical, and pharmacological characteristics. Methods of testing for the presence of ciguatera poison in tissues include intraperitoneal injection of extracts into mice, and oral administration to cats, mongooses, and puppies. This paper reports a new assay using excised frog sciatic nerve preparations.

Studies on the frequency of toxicity and the concentration of the poison in *Lutjanus bohar* from Palmyra Island show that increasing fish size is paralleled by an increase in both percentage of toxic fish and concentration of poison.

An extraction procedure using acetone and ether has been developed for removing crude ciguatera poison from fish muscle, and further work has been done on solubility characteristics.

Paper chromatography with several solvent systems has as yet proved unsuccessful as a technique for further purification of the crude toxin.

Acknowledgments

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PHARMACOLOGICAL AND CHEMICAL STUDIES OF THE VENOM OF THE STONEFISH (GENUS *SYNANCEJA*) AND OTHER SCORPIONFISHES*

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The scorpionfishes (family Scorpaenidae) include a large number of fishes of tropical and temperate seas, many of which can inflict wounds by means of stings by their venomous dorsal, pelvic, or anal spines. Effects produced by various species range from pain and swelling in some cases to death in others. Until recently little work had been reported on the pharmacological actions or chemical nature of the venoms. The venoms of several of these scorpionfishes are discussed briefly below.

Synanceja horrida (*Linnaeus*) *and S. verrucosa Bloch and Schneider* (*"Stonefishes"*)

Stonefishes (classified by Smith[1] in the family Synanciidae rather than Scorpaenidae) are found in shallow water in many areas of the tropical Indo-Pacific.[2] Each dorsal spine possesses two large venom sacs, with a duct leading to the tip of the spine. Puncture wounds may be produced by these spines, and the venom diffuses into the wound through the duct. Excruciating pain at the site occurs almost immediately after a sting, and systemic effects and death may occur. Case histories of fatal and nonfatal wounds have been reported by Smith,[3,4] Bottard,[5] Duhig and Jones,[6] and Wiener.[7] The gross behavior of several species of animals injected with stonefish venom has been described[6,8] and a hemolytic action has been reported.[9,10] More extensive studies have been carried out recently by Saunders[11,12] and Wiener.[13]

The venoms of *S. horrida* and *S. verrucosa* appear to possess essentially identical pharmacological actions as well as certain other properties. The fluid venom obtained directly from the venom sacs contains about 12 per cent protein, and the material lethal to mice is nondialyzable[11,12] and antigenic.[13] The lethal fraction is quite stable on lyophilization or storage at low temperature in the presence of glycerol.[11,12] The LD_{50} of the crude venom is approximately 200 μg. of protein/kg. on intravenous injection into mice. Injection of small doses into rabbits[11,12] produced a fall in blood pressure accompanied by an increase in respiratory rate; larger doses (FIGURE 1) caused in addition electrocardiographic changes (for example, S-T segment displacement). Injection of fatal doses produced a profound fall in blood pressure accompanied by a variety of electrocardiographic changes (often including ventricular fibrillation) and respiratory arrest. Artificial respiration was not effective in prolonging the life of the animal in most cases. Hypotensive actions have also been observed in dogs and fowl.[13]

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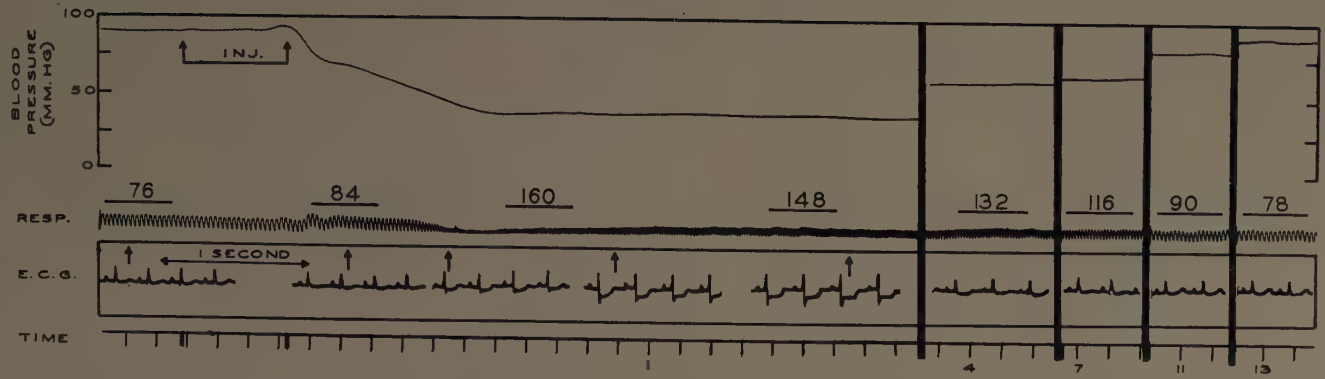

FIGURE 1. Effect of the intravenous injection (labeled Inj.) of 11.5 μg. of venom protein/kg. from *S. horrida* on blood pressure, respiration, and electrocardiogram of a rabbit. Heavy vertical lines indicate interruption of recording. Numbers above record of respiration indicate respiratory rate/min. during time interval denoted by horizontal lines beneath these numbers. Signal marks are at 5-sec. intervals, and numbers below signal marks show time (min.) after injection of venom. Arrows above ECG records (lead 1) denote point at which record was taken; time scale for ECG is indicated by horizontal arrow. Inspiration appears as downward stroke on record of respiration. Reproduced by permission of *Archives internationales de pharmacodynamie et de thérapie.*[12]

Pterois volitans (*Linnaeus*) (*Lion Fish, Turkey Fish, Zebra Fish*)

P. volitans and other species of this genus also occur in many regions of the tropical Indo-Pacific. Wounds are produced most commonly as a result of stings by the venomous dorsal spines.[14] Local effects of ths venom[5,15,16] appear to be almost identical to those produced by the stonefish (intense pain, swelling); in addition, a case involving cardiovascular collapse was described recently.[17] Saunders and Taylor[18] investigated the actions of extracts prepared from the dorsal spines of *P. volitans* and found that the effects in mice and rabbits were almost indistinguishable from those characteristic of *Synanceja* venom. The pharmacologically active substance or substances were nondialyzable and apparently protein in nature.

Scorpaena guttata Girard (*California Scorpionfish*)

S. guttata is found in rocky areas off the coast of southern and Lower California, and possesses venomous dorsal, pelvic, and anal spines.[19] Local pain and swelling are commonly experienced,[20] but serious systemic effects have not been reported. Recent work by Taylor and Saunders (unpublished) has indicated that the pharmacological actions and stability of crude extracts on storage under various conditions are similar to those of the venoms of *Synanceja* and *Pterois*.

The similarities of the actions of the venoms of the above fishes in man (for example, pain and swelling) and experimental animals suggest that the active substance or substances may be nearly identical in all cases, and that the difference in severity of wounds may be due to variation in the amount of venom entering the wound rather than to major differences in the nature of the active substances.

Methods and Results

Cardiovascular actions of the venom of Synanceja horrida. A more detailed investigation of the circulatory actions of the venom of *S. horrida* has been made recently, using venom extracts prepared from spines that had been removed from live fish (mean total length 15 cm.) in Singapore and frozen immediately. The frozen extracts were shipped in polyethylene bags in dry ice and stored subsequently at −20° C. for several months; it has been shown previously[12] that extracts prepared from such frozen spines were comparable to those prepared from fresh specimens. Extracts were prepared as described previously[12] except that the extracting medium contained 0.05 *M* sodium phosphate buffer (*p*H 8.0) in addition to 0.9 per cent NaCl. Glycerol was added to a final concentration of 40 per cent (v/v)[12] and the solutions were stored at −20° C. Extracts were bioassayed by intravenous injection into white mice (mean weight 22 gm.), and no detectable change in potency or action occurred in the 5-month period during which the following experiments were carried out.

Rabbits (mean weight 2.3 kg.) were anesthetized with 1.0 to 1.5 gm. of urethane/kg. intraperitoneally. Blood pressure was recorded from a carotid or femoral artery, and electrocardiograms from standard limb leads. Pressor responses to levarterenol (norepinephrine), carotid occlusion, and asphyxia with

N_2 were investigated. In some experiments both vagi were sectioned and the central end of the right vagus stimulated at approximately 4 V. and a frequency of 50/sec., with square wave pulses of 1 to 5 msec. duration for 10 to 15 sec. The right depressor nerve was sectioned in some of these bilaterally vagotomized animals, and stimulated centrally at 0.5 V. and a frequency of 50/sec., with 0.2 msec. pulses for 10 sec. Responses to these conditions were determined before and after intravenous injection of venom.

Data from these experiments are given in TABLE 1. It is apparent that the venom of *S. horrida* did not alter the responses to any of these maneuvers. The unchanged response to levarterenol after administration of venom indicates that the latter does not possess adrenergic-blocking activity. Unchanged responses to carotid occlusion, asphyxia, and central stimulation of the cut vagus or depressor nerves suggest an absence of effect upon the sympathetic nervous system outflow to blood vessels, as the blood pressure responses to these maneuvers are at least in part mediated through such a mechanism. There are, in addition, no indications from these experiments of an action upon central nervous system vasomotor mechanisms involved in these responses.

TABLE 1
EFFECT OF VENOM OF *SYNANCEJA HORRIDA* UPON CERTAIN BLOOD PRESSURE RESPONSES IN THE RABBIT

Experimental condition	Blood pressure response prior to venom (mean values) (mm. Hg)	Blood pressure response after venom (mean values)* (mm. Hg)
Levarterenol (2 μg./kg. I.V.)	28 (increase)	30 (increase)
Carotid occlusion for 30 sec.	20 (increase)	18 (increase)
Asphyxia for 15 sec.	35 (increase)	38 (increase)
Stimulation right vagus centrally	21 (decrease)	19 (decrease)
Stimulation right depressor nerve centrally	18 (decrease)	16 (decrease)

* The blood pressure decreased after injection of the venom to a mean value that was 72 per cent of the control pressure, except in the carotid occlusion experiments in which smaller doses of venom were used (blood pressure decrease to mean value of 85 per cent of control). The tests listed in the left column above were carried out when the blood pressure had reached a minimum following injection of venom.

Extracts were also injected intra-arterially into the blood supply of a hind limb of a rabbit and the flow rate measured. The injection was made with a constant injection apparatus at a rate that gave an arterial blood concentration equal to that calculated to be present following intravenous injection of amounts sufficient to lower systemic blood pressure by approximately one half. The flow rate was unchanged following injection of venom in these amounts. Lower intra-arterial concentrations of venom were also without effect, while concentrations about ten times greater produced only vasoconstriction. It is clear, therefore, that the hypotension is not due to a direct vasodilatation of the blood vessels of skeletal muscle (unpublished work by P. R. Saunders, S. Rothman, T. Chin, and V. Medrano).

The possibility that the hypotension might be due to a depression of force of contraction of the heart was investigated by simultaneous recording of blood pressure and contractile force by means of a strain-gauge arch[21] sewed to the right ventricle of open-chest rabbits. Intravenous injection of venom in amounts sufficient to decrease the blood pressure to about two thirds the pre-injection value led to some decline in force of contraction; this decline in force occurred, however, subsequent to the initial fall in blood pressure, suggesting that it was, at least in part, secondary to a decreased venous return as a result of the blood pressure decrease (unpublished data by Saunders, *et al.*; *op. cit.*). The initial fall in blood pressure in the absence of changes in contractile force suggests that at least the early phase of hypotension is due to peripheral vasodilatation rather than to a decrease in cardiac force. It should be noted, however, that other effects on the heart were sometimes seen at these doses: auricular and ventricular extrasystoles sometimes occurred, and slightly greater doses (sufficient to decrease the blood pressure about one half) have been shown previously to cause T wave changes and S-T segment displacement (FIGURE 1) indicative of cardiac injury. In addition, injection of fatal doses sometimes produced ventricular fibrillation before the blood pressure had decreased markedly. It is possible, therefore, that cardiac effects may also be involved in the hypotension produced by larger doses of venom.

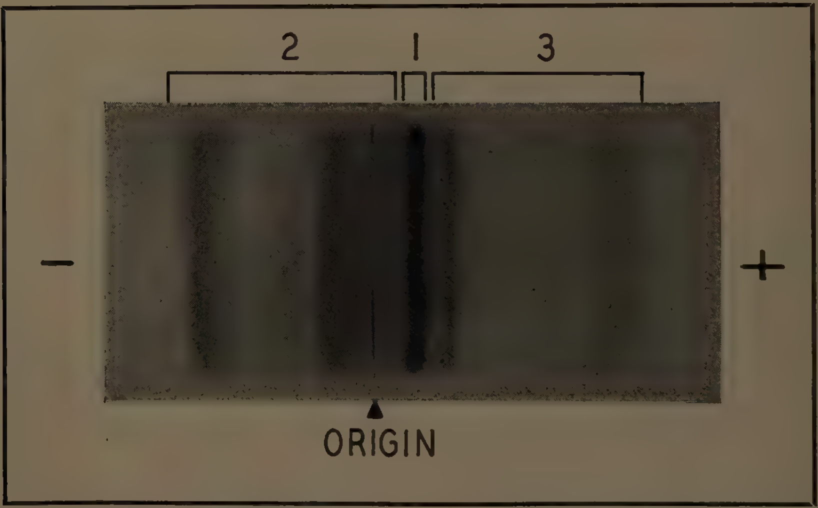

FIGURE 2. Starch gel electrophoresis of venom of *S. horrida* (see text). Fraction "1" above was lethal to mice, but no effects were produced in mice with the combined fractions labeled 2 and 3.

Starch gel electrophoresis of the venom of Synanceja horrida. *S. horrida* venom has been subjected to starch gel electrophoresis[22] in an attempt to determine the number of protein fractions present and their biological activity in mice. A 12.5 per cent starch gel was prepared in sodium phosphate buffer (pH 8.0, μ 0.1) containing 10 per cent glycerol. Undiluted venom (0.025 ml.) from spines frozen for 2 to 6 months was applied to a small piece of filter paper, which was inserted into a slit in the gel. Electrophoresis was carried out at a potential gradient at 6 v/cm. at 5° C. for 6 hr.; the gel was then sliced and one portion stained with Naphthalene Black 12B-200 to locate the bands. Corresponding regions of the unstained gel were cut out, frozen at −20° C. for 1 hour, and the spongelike structure centrifuged after thawing. The fluid portion was bioassayed in mice and its nitrogen content determined by a modified Nessler procedure.[23] A typical separation pattern is shown in FIGURE 2. A lethal effect in mice was obtained only with the fraction labeled 1 in the figure; intravenous injection of this fraction produced in mice symptoms apparently identical with those obtained with fresh venom.[12] No effect was produced in mice by eluates of the areas labeled 2 and 3, in both of which several bands were present; these latter fractions were inactive at doses of nitrogen/kg. that were 10 or more times as great as those that were lethal following injection of fraction 1. Only small amounts of eluted fraction 1 were available for bioassay; however, intravenous administration of this fraction at a dose of 15 μg. of nitrogen/kg. killed 6 out of a total of 11 mice injected, indicating an LD_{50} near this value. The material in crude venom extracts that is lethal to mice is nondialyzable,[12] and it is therefore presumed that the above active fraction is a protein or mixture of proteins.

Summary

The cardiovascular actions of the venom of the stonefish *Synanceja horrida* were investigated in anesthetized rabbits. The most characteristic response to small to moderate doses of the venom was a decrease in blood pressure. The hypotension was apparently primarily due to a peripheral vasodilatation, although a decrease in force of contraction of the heart was observed soon after the blood pressure began to fall. The vascular bed(s) in which vasodilatation occurred was not determined, but vasodilatation did not occur in the hind leg of the rabbit following intra-arterial injection of venom.

Stonefish venom did not block the pressor responses to levarterenol, carotid artery occlusion or asphyxia. Depressor responses to electrical stimulation centrally of the cut right vagus or right depressor nerves were also not blocked. These experiments indicate an absence of adrenergic-blocking action, and suggest that blockade of sympathetic ganglia also did not occur. Failure of the venom to modify blood pressure responses to stimulation of the above afferent pathways also indicates that the hypotensive action is not due to depression of those parts of the central nervous system involved in these vasomotor reflexes.

Stonefish venom has been separated by starch gel electrophoresis into a number of components, and a lethal action in mice could be demonstrated with only one of these fractions.

Acknowledgment

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VENOM OF THE WEEVERS *TRACHINUS DRACO* AND *TRACHINUS VIPERA**

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The weevers, members of the piscine family Trachinidae, are small marine fishes that appear to be confined to the eastern Atlantic and Mediterranean coasts. The name "weever" is probably derived from a corruption of the Anglo-Saxon "wivre," meaning "viper." The lesser weever, *Trachinus vipera* (FIGURE 1), reaches a total length of about 13.5 cm.[1] Of 167 specimens examined during the course of the present investigation, the longest measured 12.1 cm., the shortest, 6.5 cm., and the mean total length was 8.6 cm. These fish are found in large numbers in the shallow waters of certain offshore sandy grounds along the southeast English coast, in the continental southern North Sea, and along the coasts of the English Channel and Mediterranean Sea. They are often taken by inshore vessels engaged in shrimping. However, as they are of little commerical value no figures are maintained on the yearly catch or on the numbers landed at various ports.

The greater weever, *Trachinus draco* (FIGURE 1), is said to reach a total length not exceeding 45.72 cm.,[2] although Maretic[3] reports that these fish may reach a total length of about 50 cm. Of 36 specimens examined by the senior author, the longest was 44.6 cm., the shortest was 11.1 cm., and the mean total length was 28.4 cm. These fish are usually taken in the waters of the Kattegat and Skagerrak, the southern North Sea, and the English Channel, off the south and west coasts of England, in the Bay of Biscay, around the island of Madeira, along the Atlantic coast of North Africa, and in the Mediterranean and Adriatic Seas. They are rare off County Cork, Ireland,[4] and north of Bergen, Norway.[5] Most of them are caught by deep water trawlers and coastal cutters. When the catch is large enough to warrant marketing, these fish command a good price and appear to be well esteemed in some North Sea ports.

According to Roessingh (personal communication, 1960), 3500 kg. of these fish were landed at ports in the Netherlands during 1958. In tonnage the greater weever ranked 48th among the 62 different species of fishes brought into Dutch ports during that year. Cole (personal communication, 1960) reports that landings of weeverfishes in recent years at Lowestoft, England, have been negligible, consisting of only a few stone a year. The practice of removing the venomous dorsal and opercular spines before marketing does not appear to be well established in England, Holland, or France at the present time.

Medical Significance

Injuries inflicted by weeverfishes are not uncommon.[3,6-15] Stings by the lesser weever usually occur when the unfortunate bather treads upon the dorsal

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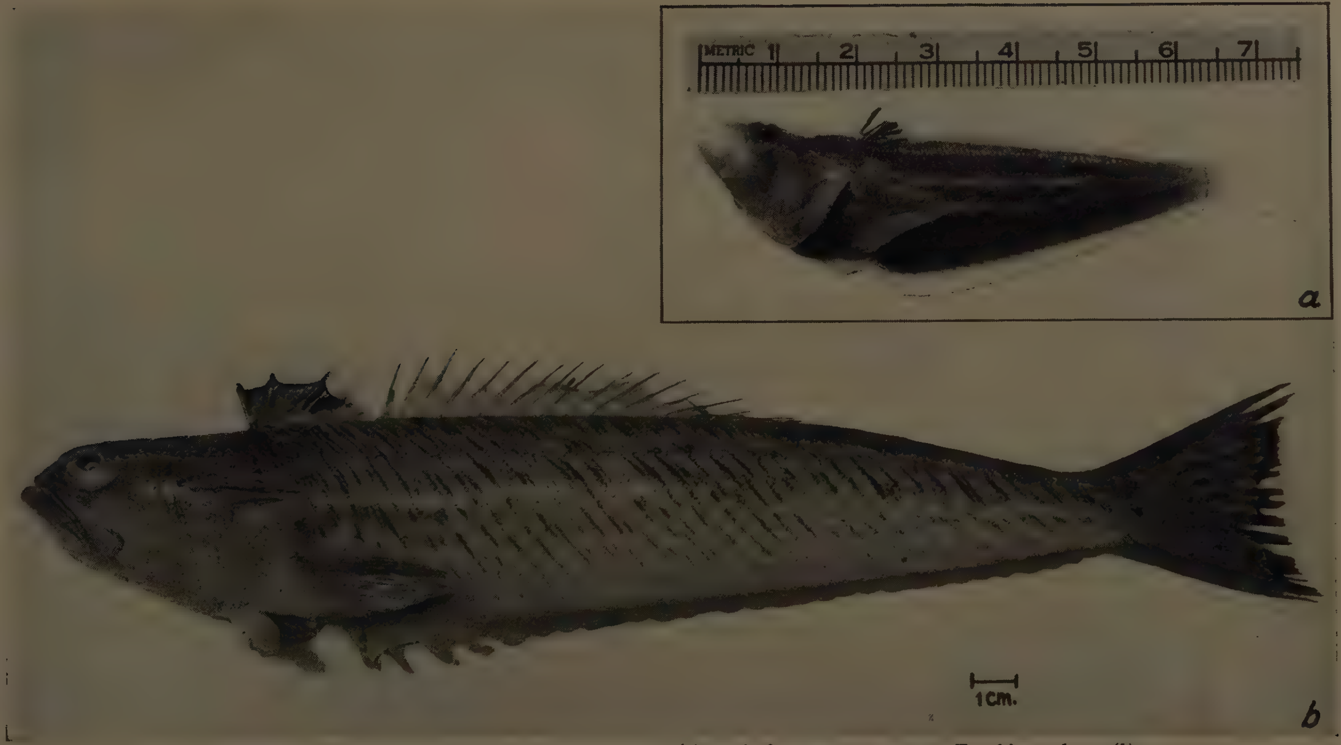

FIGURE 1. The lesser weever *Trachinus vipera* (*a*) and the greater weever *Trachinus draco* (*b*).

spines of one of them while wading on sandy grounds inhabited by them, or when a fisherman mishandles the fish while dislodging it from a net. Occasionally these fish become entangled in seaweed during trawling operations and are taken aboard unknowingly. The lesser weever often buries itself in the sandy or muddy ocean bottom and may remain there motionless with only its erect dorsal spines projecting above its camouflage. When the fish is stepped upon, the integumentary sheaths surrounding the venom-containing tissues of the spines are torn and the venom escapes into the victim's tissues. Such injuries are frequently reported along the Devon and Cornwall coasts of England.[16] In an interesting case history reported by Halstead[14] a lesser weever was said to have aggressively attacked a skin diver, driving its fins into the victim's right jaw. Most stingings by these fish, however, are inflicted on the hands or forearms of the shrimp fishermen of the North Sea.

The greater weever may also bury itself in the sand. However as it does not customarily invade very shallow waters it is not usually implicated in stinging bathers. Most injuries inflicted by *T. draco* are incurred by fishermen removing it from their nets or set lines. According to Evans[15] the greater weever may employ its venomous spines as offensive weapons, "attacking any object approaching it with the precision of a fighting cock."

A weeverfish may inflict either a single or multiple puncture-type wound. Persons stung by these fishes report having received a sharp, immediately painful stab. The pain is described as intense or excruciating; it increases in severity during the first 20 to 50 min. following the injury and may persist for 16 to 24 hours if treatment is not undertaken. The pain is out of proportion to that which might be produced by a similar wound from a nonvenomous fish. During the initial 5 to 10 min. following the stinging the pain is usually confined to the area about the stab, but subsequently it spreads to involve the entire affected extremity. Axillary pain is common in patients stung on the hand by weeverfishes. The pain is more severe than that observed following stingings by stingrays. As noted by Halstead[14] the pain can be so severe that a victim stung by one of these fishes while in the water may experience difficulty in reaching shore. One of us (F.E.R.) has received similar reports from swimmers who had been stung by a weeverfish while bathing in the surf. It seems likely that it is the excruciating pain rather than true muscular paralysis that is responsible for the victim's motor incapacity. A similar incapacity has been reported previously following stingray injuries.[17]

The amount of bleeding from the puncture wound appears to be about what one might expect from a similar nonvenomous injury. The degree of swelling about the wound varies, although some swelling appears to be a constant finding. An increase in the skin temperature of the involved tissues is common. The tissues adjacent to the wound often appear discolored. Examination usually reveals a definite necrosis, and sloughing of these tissues is reported to occur.[14] In most cases the symptoms and signs provoked by the venom are local manifestations. However, there is both experimental and clinical evidence to indicate that the venom may produce systemic changes. This evidence has been reviewed elsewhere.[11,14] Deaths from weever stings are rare.

The treatment of weeverfish stings does not appear to be well established.

Fishermen in the North Sea and Mediterranean Sea still use many different remedies, including the local application of alcohol, ammonia, carbolic acid, fish liver, formaldehyde, gasoline products, potassium permanganate, tobacco juice, wine, vinegar, or hot water. Roessingh (*op. cit.*) states that some of the older fishermen along the North Sea coast seriously recommend "burning the devilish weever, swear and pray." It would appear from the several cases seen by F.E.R. that the use of hot water in the first aid treatment, as recommended for stingray injuries,[17-19] seems advisable. Further medical care for this type of injury is discussed in these papers.

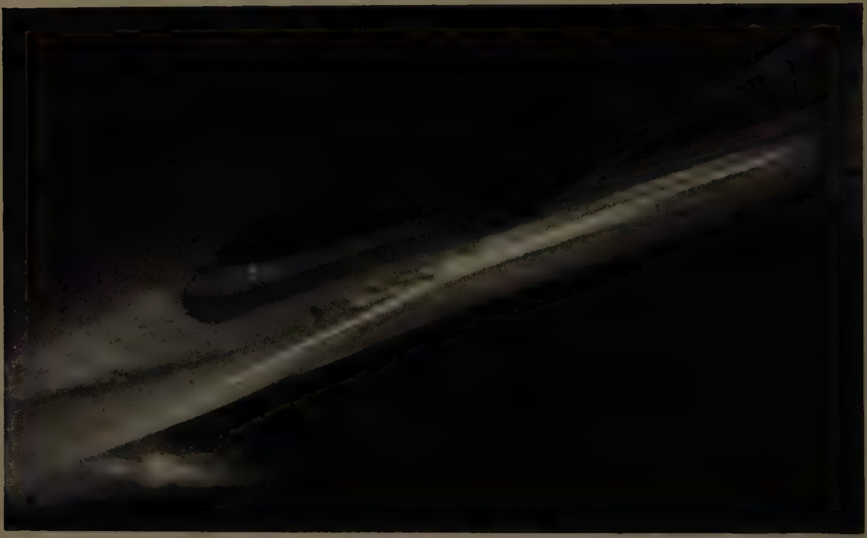

FIGURE 2. Opercular spine of *T. draco*, showing pin inserted into superior groove. Note the conic cavity at the base of groove.

Venom Apparatus

Opercular spines. The venom apparatus of these fishes consist of two opercular spines, five to eight dorsal spines, and the tissues contained within the integumentary sheaths surrounding the spines. The two dentinal opercular spines extend caudally and very slightly downward from near the upper margin of each operculum. They are firmly attached to the operculum for the proximal one third of their length, and lie free and superficial to the posterior portion of the operculum for the distal two thirds.

FIGURE 2 shows a pin inserted into the mid-portion of the deep groove along the superior margin of the left opercular spine of *T. draco*. A similar groove exists along the inferior margin. The portion of the spine not attached to the operculum measured 18 mm. Within the superior and inferior grooves, and in the two conic cavities into which they enter at the base of the spine, lies most of the spongy glandular tissue that produces the venom. The spine is covered by an integumentary sheath that, when ruptured, allows the toxin to escape from the venom-laden cells. The microscopic anatomy of the tissues associated with the opercular and dorsal spines of the weevers has been reported by Schmidt,[11] Byerley,[20] Parker,[21] Borley,[22] and Halstead and Modglin.[23] Halstead and Modglin found no significant qualitative morphological differences between the venom organs of *T. draco* and *T. vipera.*

Dorsal spines. The five to eight dorsal spines are enclosed within individual integumentary sheaths connected by thin interspinous membranes. Dorsal spines were examined in 16 *T. draco.* The following description is based on an examination of the 7 dorsal spines of a greater weever measuring 38.4 cm. in total length.

Spines 1 through 7 measured 21, 26, 25, 19, 13, 9, and 5 mm., respectively. The first spine curves slightly caudally from its articulated base to the tip. It has a prominent anteromedian ridge flanked on one side by a groove that extends from the tip of the spine toward the base for a distance of 14 mm. On the opposite side of the ridge a similar groove is seen at the tip of the spine. This lateral groove extends toward the base for about 6 mm., then passes more posteriorly, terminating about 14 mm. from the tip. At its point of termination the groove lies on the posterior surface of the spine. At the point where the groove begins to extend posteriorly, a thin ledge of dentine arises, becoming the anterior margin of the groove. This ledge extends to the base of the spine and contains a shallow groove near its termination. The lateral groove on this side is broader and deeper than that on the opposite side.

The second dorsal spine has a prominent anteromedian ridge flanked on either side by deep grooves that extend from the tip toward the base for a distance of 14 mm., at which point the sides of the grooves meet, closing the grooves. The closed grooves extend to the base of the spine as fine depressions in the dentine. At about 9 mm. from the tip on one side and 12 mm. on the other, dentinal ledges arise from the sides of the anteromedian ridge and extend to the base of the spine. The posterior surfaces of these ledges become the anterior margins of the lateral grooves. On one of these ledges near the base of the spine is a shallow groove much like that seen in spine 1. The lateral grooves in the second spine are deeper and broader than those observed in the first spine, reaching their greatest size about 10 mm. from the tip.

The third dorsal spine is very similar to the second except that the lateral groove on one side terminates 15 mm. from the tip, while on the other side it continues from about the same point to the base of the spine as a fine depression in the dentine. The fourth spine is very similar to the first. One of its lateral grooves terminates on the posterior surface of the spine. The fifth spine has a prominent anteromedian ridge flanked by two lateral grooves that extend from the tip toward the base for a distance of 6 mm. No other grooves or ledges are noted. The sixth spine has a lateral groove of 1.5 mm. on one side and no groove on the other. The seventh dorsal spine is devoid of grooves.

From the above description it appears that there may be considerable variation in the dorsal spines of a single weeverfish. Also, the grooves in individual spines may not be symmetrical with respect to location, size, and number. This description certainly does not conform with that given by Halstead and and Modglin.[23] However, meticulous examination of a number of dorsal spines revealed some that fit the description presented by these authors. During the examination of these spines, some were found that had several deep grooves between the anteromedian ridge and the primary lateral groove on one side. These additional grooves did not extend the full length of the spine. In some spines there were additional grooves lateral and posterior to the primary lateral groove; occasionally, spines were found with grooves on the posterior surface or on the anteromedian ridge.

Materials and Methods

Preparation of crude venom extract. The opercular and dorsal spines with their intact integumentary sheaths were severed from live *T. draco* and immediately refrigerated at −25° C. The spines were maintained at this temperature, or lower, until several minutes before the experimental procedure, at which time the venom was extracted. For some experiments the opercular and dorsal spines of *T. vipera* were used, or the tissues of the two fishes mixed together. The integumentary sheath and all tissues lying beneath it and adjacent to the spines and within the lateral grooves were removed under microscopic observation and macerated with 1.0 ml. of 0.9 per cent saline in a tissue homogenizer until a homogeneous suspension was obtained. It was realized that most of the venom would be found in the soft spongy tissues of the grooves and conic cavities. However, in the removal of the integumentary sheaths and underlying tissues, some venom escapes and can be found in the tissues adjacent to the venom-producing cells. For this reason all tissues connected with the spines were removed for the preparation of the venom solution.

Two ml. of saline was then added and the suspension centrifuged at 3000 rpm for 3 min. The supernatant was poured off and the residue returned to the tissue homogenizer, where it was subjected to further extraction in the presence of 1.0 ml. of saline. The re-extracted portion was again centrifuged and the supernatant added to that obtained from the first centrifugation. The decanted solution was brought up to a total volume of 4.0 ml. with saline, and represents an extract from approximately 3.9 mg. of lyophilized tissue.

In the studies of the chemistry of the venom, extracts were prepared with distilled water in place of saline. All chemical studies were made from a single pool of the venom solution. This pool was derived from the spines of 86 *T. vipera* and 6 *T. draco*.

Chemistry studies. The percentages by weight were determined for carbon, hydrogen, nitrogen, sulfur, and phosphorus. The carbon and hydrogen contents were calculated following combustion of a sample of the pooled material in a measured volume of oxygen under controlled pressure. The yield of carbon dioxide and water were determined gravimetrically and the percentages calculated.[24] Nitrogen determinations were made using a modification of the Dumas technique.[24]

Sulfur determinations were made after the microbomb method of Elek and Hill.[25] The percentage of total phosphorus was obtained using the method proposed by Fiske and Subbarow[26] following digestion of the organic matter in the sample with 5 normal sulfuric acid.

Determinations were also made of the moisture content, total lipids, and total inorganic matter. Total protein was calculated by multiplying the nitrogen value by 6.25. Moisture content was determined by drying a fresh sample of the tissues to a constant weight at 105° C. Total mineral matter was found by ashing the sample at 600° C. to a constant weight and calculating the percentage of inorganic matter by weight difference.

A freshly prepared venom solution was divided into 2 portions. One portion was dialized in Visking cellulose casing at 4° C. for 20 hours, and the second portion maintained at 4° C. for the same period of time. The LD_{50} of the dialysate and of the casing content was determined in 20-gm. Swiss albino mice. The results were compared with those obtained with the control solutions.

Neuromuscular transmission study. Studies were made of the effects of the venom on the phrenic nerve-diaphragm preparation after a method originally described by Bülbring,[27] modified by Holmes *et al.*,[28] and Jenden,[29] and further modified for studies on venoms by Russell and Long[30] as follows:

A 1-cm.-wide segment of diaphragm from the left side was removed, along with the phrenic nerve, from guinea pigs weighing 150 to 200 gm. The diaphragm, cut parallel with the muscle fibers, was left attached to a slip of the intercostal muscle mass to facilitate attachment at the lower muscle electrode, and a small segment of the muscle on the dorsal side of the tendon was retained for attachment of the thread tied to the writing lever. The phrenic nerve was cut in the neck and dissected free to its termination at the diaphragm. Removal of the diaphragm and phrenic nerve was accomplished in less than 100 sec.

The preparation was mounted on a lucite electrode support similar to that described by Holmes *et al.*[28] The intercostal muscle mass was attached to the platinum lower muscle electrode (positive) with a rubber band. The tendinous portion of the diaphragm did not contact the upper muscle electrode (negative). This electrode is a mass electrode and sits at the level of the meniscus of the bath. The thread attached to the tendinous end was threaded through the upper electrode and tied into a carefully balanced writing lever that was charged through an Electrex unit to record on Electrex charting paper. Contraction of of the muscle caused an upward deflection of the writing arm. The phrenic nerve was threaded through the 2 platinum nerve-electrodes that lay just below the upper muscle electrode. Stimuli were applied alternately to the muscle and to the nerve at the rate of 1 stimulus every 20 sec. to each segment. In the case of the nerve, the discharge from a 0.02 mFd condenser charged to approximately 6 volts was used; to stimulate the muscle directly, a 2.0 mFd condenser charged to 40 volts was used. Maximal contractions were obtained with these stimuli.

Krebs solution, as modified by Jenden[29] (and described in a personal communication, 1959), made up the day of the experiment, was used in all preparations. Water was triply distilled. The tissue bath, which had a capacity of 25 ml., was maintained in a constant-temperature apparatus at 37° C. The bath was fitted with an oxygenator and a vacuum-emptying device so that the chamber could be evacuated and refilled in less than 7 sec. The Krebs solution was continuously oxygenated with a gas mixture containing 95 per cent O_2

and 5 per cent CO_2. Both the solution in the bath and the stock solution were oxygenated and maintained at 37° C. Each preparation was allowed to equilibrate during the hour preceding the experiment. Foaming was controlled with a silicone antifoam emulsion.

Cardiovascular study. Studies were made of the effects of the venom on the cardiovascular system using a modification of methods previously described.[31,32] In five adult cats under pentobarbital hypnosis a common carotid artery and femoral vein were cannulated for measurement of arterial and venous pressures. Bipolar electrodes were placed in the skull over the frontal and parietal lobes, bilaterally. Conventional limb, unipolar extremity, and precordial leads were affixed. All electrodes were connected to a Medcraft model D, 8-channel electroencephalograph. Respirations were recorded by means of a cantilever pneumograph[33] fed into the electroencephalograph. In one animal intermittent artificial respiration was carried out by positive-pressure breathing through a tracheal cannula. Cisternal pressure was measured through a 20-gauge needle fixed in the cisterna cerebello-medullaris and connected to a modified Brodie tambour in parallel with a water manometer. The tambour activated a wire strain-gauge transducer that modulated the amplitude of a 3-cycle carrier that fed the response into the electroencephalograph. A femoral vein was cannulated for the subsequent injection of the venom. All animals were heparinized.

TABLE 1
ANALYSIS OF EXTRACT OF TISSUES OF OPERCULAR AND DORSAL SPINES OF WEEVERFISHES

Elemental analysis	Per cent by weight	Proximate analysis	Per cent
Carbon	22.8	Moisture	41.5
Hydrogen	3.5	Protein	21.7
Nitrogen	6.9	Lipids (ether soluble)	3.5
Phosphorous	2.2	Inorganic matter	12.9
Sulfur	0.0	Carbohydrate (calculated)	20.4

In one dog, a common carotid artery was cannulated for the measurement of arterial pressure. A standard single-lumen Cournand cardiac catheter was placed in the right femoral vein and threaded under fluoroscopy through the common iliac vein, postcava, right atrium, right ventricle, and then into the pulmonary artery. The carotid and pulmonary artery pressures were transmitted to Statham pressure transducers and recorded on a Sanborn Polyviso recorder.

Results

Chemistry studies. The freshly prepared extract is clear, and gray in color. It has a fishy taste and ammoniacal odor. Its *p*H is 6.78. The results of the elemental and proximate analysis are shown in TABLE 1. In some respects the findings are similar to those observed for the venom of the stingray.[34]

Assays of the dialyzed venom solution and the undialyzed controls indicated that the lethal portion remained in the sac after the 20-hour dialysis.

Neuromuscular trasnmission study. The effects of the venom were studied in 10 preparations. FIGURE 3 shows the changes produced in the tracings from 3 nerve-diaphragm preparations in which different amounts of the venom were added to the bath. With 1.0 ml. of the venom solution there was no change in either the directly or indirectly elicited contractions. Two ml. of the extract produced a slight shortening of the muscle, and a very gradual depression of

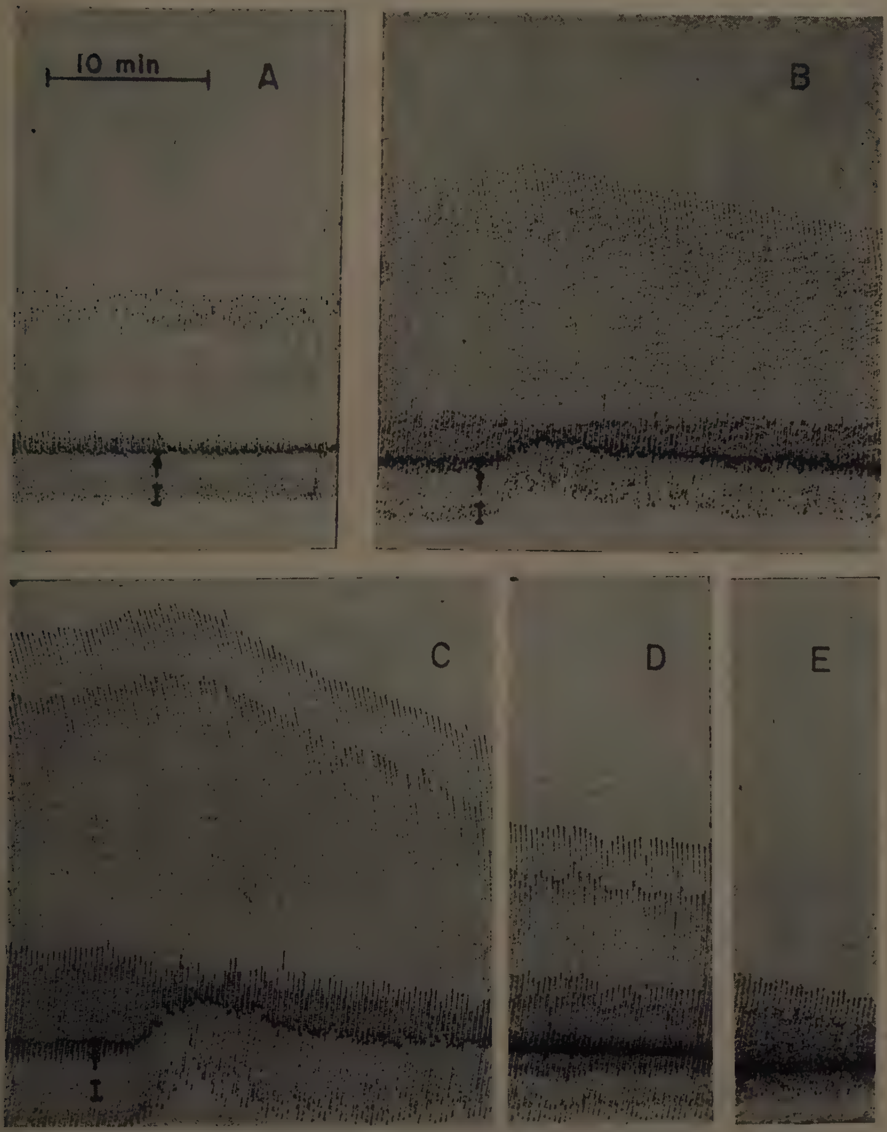

FIGURE 3. Effects of weeverfish venom on the phrenic nerve-diaphragm preparation. A, 1.0 ml.; B, 2.0 ml.; C, 4.0 ml.; D, 1 hr. following injection of 4.0 ml. of venom; E, 2 hrs. following injection of 4.0 ml. of venom.

both the directly and indirectly elicited contractions over the 4-hr. observation period. When 4.0 ml. of the extract was added there was a shortening of the muscle and a gradual depression of both excursions over the 2-hr. observation period. However, at no time did the indirectly elicited contractions appear to decrease more rapidly than the directly elicited contractions. With larger doses there was a slightly greater initial shortening of the muscle and a more rapid decrease in the height of both the directly and indirectly elicited contractions. However, the fall in the height of the two excursions was parallel at all times.

It appears that weever venom has no remarkable effect on the neuromuscular junction. The initial shortening of the muscle can be attributed to its irritation by the venom. The subsequent gradual depression of the two excursions indicates that while the venom has an effect on both muscle and nerve, it is not marked, and it is no greater than one would expect following reduction of the oxygen supply to the preparation, or alterations in the balance of electrolytes in the bath.

Cardiovascular study. FIGURE 4 shows the changes in the systemic arterial pressure, venous pressure, cisternal pressure, cardiac rate, electrocardiogram, electroencephalogram, and respiration produced by a lethal dose of the venom, approximately 3 to 8 ml. of the venom solution.

The precipitous fall in systemic arterial pressure began within 20 sec. of the end of the injection in all preparations. In two animals there was an initial fall of 35 mm. of mercury during the first 30-sec. period. A constant pressure was then maintained for the next 10 sec. Following this the pressure again declined rapidly, reaching its lowest level, 20 mm. of mercury, 1 min. following the injection. This staircase effect was similar to that observed following the injection of a lethal amount of stingray venom.[35] The rise in systemic venous pressure was observed in 3 of the 5 preparations. In two of the animals it occurred within 30 sec. of the initiation of the fall in arterial pressure. In one preparation it did not occur until 90 sec. after the initial fall in arterial pressure. In the remaining two preparations the venous pressure rose as the arterial pressure approached its lowest level, and then declined slowly, producing a curve somewhat parallel to the arterial pressure curve.

In general, the cisternal pressure tracings resembled the venous pressure tracings. It appeared that the rise in cisternal pressure began before the rise in venous pressure, and reached its peak after the venous pressure had begun to adjust at its maximum level.

The electrocardiograms were abnormal and unpredictable. Two distinct effects were noted: disturbances in rhythm and ischemic injury patterns. The usual initial and immediate response was bradycardia, prolongation of the PR interval with characteristic patterns of first degree, second degree, and, in two cases, third degree auriculoventricular block. Sinus arrest following a second degree block was seen in the animal. In two animals there was an almost immediate ST, T wave change, indicative of ischemia or true muscle injury. The late effects were too variable for interpretation.

The electroencephalograms usually reveal some flattening of all activity within 15 to 75 sec. following injection of the venom. In two animals there was

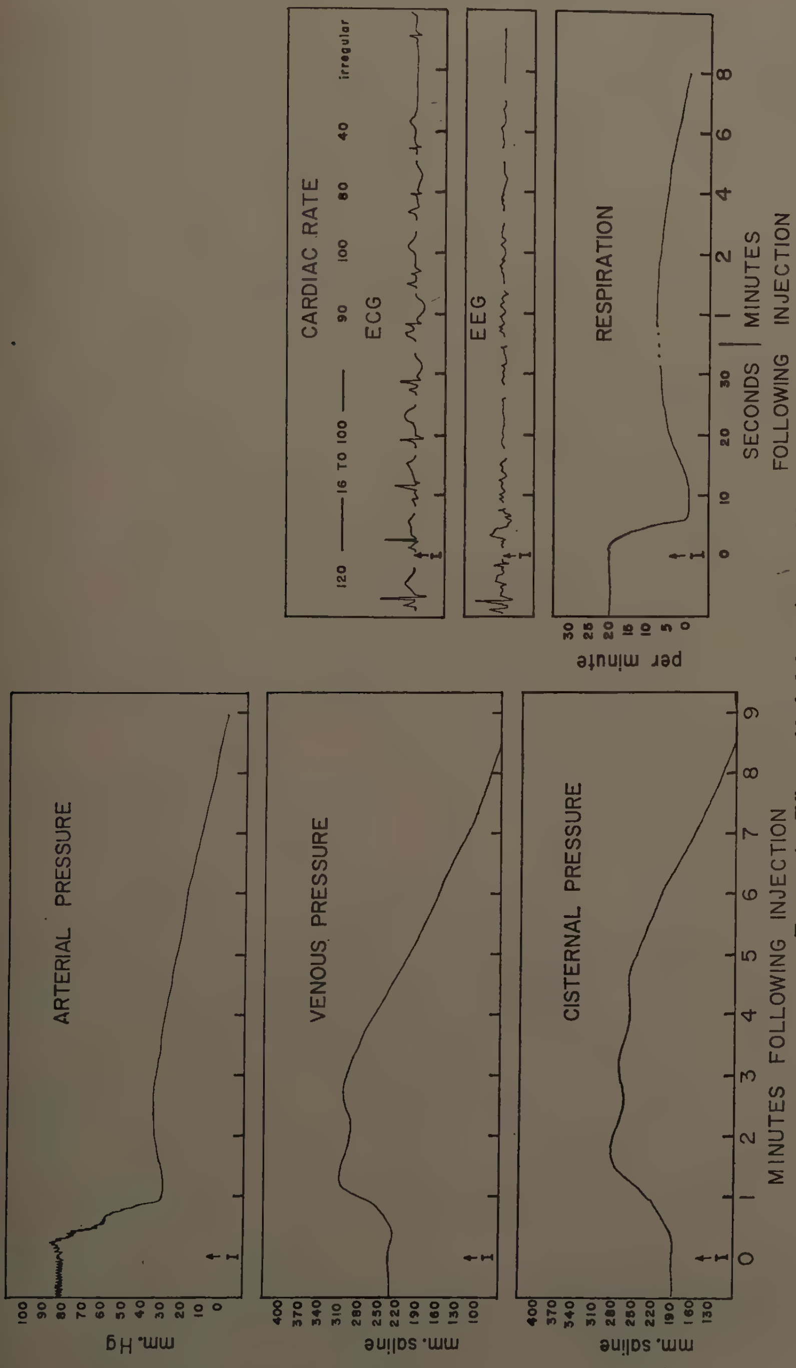

Figure 4. Effects of lethal dose of weeverfish venom.

a marked lessening of spindle burst activity. The frequency of the waves forming the burst was decreased and the waves between bursts were of lesser amplitude. In one animal random slow waves of varying potentials were observed in the frontal-parietal areas. Following the initial 20-sec. period there was a slight increase in the amplitude of all waves. Subsequently, the amplitude of the waves gradually lessened until the tracing became a straight line and the animal died shortly thereafter.

The effects of the venom on respiration were marked. In two animals respiration stopped for 10 to 40 sec.; in inspiration, within 10 sec. of the end of the injection. The subsequent respirations were irregular, often gasping in nature, and of a lesser frequency. In one animal respiratory movements decreased in frequency from 18 to 9 during the first minute, and then decreased slowly until complete apnea occurred 16 min. later. In the cat given the smallest dose of the venom, respiratory movements ceased for 10 sec. following the injection, then increased in frequency from 16 to 28 for a period of 4 min., gradually decreasing until death at 28 min. In all animals the regular respiratory movements following injection became more shallow and more abdominal in type.

The dog in which a common carotid artery was cannulated and the pulmonary artery catheterized for the measurement of arterial pressures exhibited changes similar to those observed in the cats. During the 2-min. period immediately following the injection of a lethal dose of the venom, the pressure in the carotid artery fell rapidly from 168 to 70 mm. of mercury. An initial increase of 20-sec. duration in the pulmonary artery pressure and pulse pressure was observed. The pressure then fell at a rate somewhat slower than that in the carotid artery. Respiratory stand-still of 20-sec. duration occurred 10 sec. following injection of the venom. Subsequent respirations were irregular and gasping in nature. In spite of the induction of intermittent artificial respiration carried out with a positive pressure breathing apparatus the dog died 20 min. following the injection.

Discussion

The limited number of weeverfishes available made it difficult to perform more quantitative zootoxicological studies on the venom. Determinations of the LD_{50}, the minimal cardiovascular response, and certain chemical properties of the toxin had to be deferred. During the cardiovascular study an attempt was made to elicit minimal responses. However, the venom appeared to be more lethal than expected, with the result that the changes described were irreversible and led to death. Small amounts of the venom may produce quite different changes in the parameters of the cardiovascular system, as demonstrated with varying doses of the venom of the stingray.[31,35,36]

Few data were obtained on the relative potencies of the venom following different methods of extraction. While 4.0 ml. of the venom solution contained approximately 3.9 mg. of lyophilized tissue, the amount of venom present was indeterminable, as the material subjected to extraction represented, in addition to the venom glands, all the tissues adjacent to the spines. Subsequent investigations with extracts of the venom glands alone indicate that only a portion of the venom solution described in this report contained the active toxin. Studies are in progress with the more concentrated extracts.

The effects of weeverfish venom on the mammalian cardiovascular system and neuromuscular junction are quite similar to those of stingray venom. The precipitous fall in systemic arterial pressure, concomitant with changes in the pulse pressure, cardiac rate, pulmonary arterial pressure, venous and cisternal pressures, respiration, and the electrocardiogram and electroencephalogram, resembles that seen following injection of lethal amounts of stingray venom. Both venoms appear to exert their most deleterious effects on the cardiovascular system and, in particular, on the heart. The electrocardiograms demonstrate that the venom can produce both changes in rhythm and injury to the heart muscle. The fall in pulmonary arterial pressure indicates either a failure of the heart to maintain an effective stroke volume, or a decrease in pulmonary resistance. The latter seems unlikely; preliminary studies of pulmonary artery blood flow using a gated sine-wave electromagnetic flowmeter[37] indicate that the blood flow in this vessel is reduced during the period of decreased pressure. The findings of lowered pulmonary arterial pressure and flow, a decrease in cardiac rate with various degrees of auriculoventricular block, and evidence of heart muscle injury may be interpreted to mean that some degree of cardiac failure is probably responsible for the fall in systemic arterial pressure and the rise in venous and cisternal pressures.

The depression in central nervous system electrical activity can be attributed to an ischemic anemia produced by the lowered systemic arterial pressure. The wave amplitude increases as the arterial pressure rises, indicating that the changes in the electroencephalogram are probably dependent on the blood pressure changes. While the present investigation indicates that the cardiovascular alterations are probably responsible for the marked changes in the electrical activity of the brain, it is possible that the venom may have a direct effect on the central nervous system.

It is always difficult to interpret alterations in the respiratory cycle in the presence of profound changes in the parameters of the cardiovascular system. Experiences with stingray venom showed that respirations could be affected by the choice of test animal employed, the kind and dose of anesthetic given, and the amount of venom injected. In the cat, under pentobarbital anesthesia (35 mg./kg.), small amounts of stingray venom produced either no change in respiration or an increase in the respiratory rate and somewhat more superficial respiratory movements. With increasing doses the changes varied. After the injection some animals exhibited a short period of apnea, followed by either an increase or a decrease in the respiratory rate, while others showed a marked slowing of respiratory movements without an initial period of apnea. When lethal amounts of stingray venom were given there was a period of apnea followed by irregular, gasping respirations that decreased in frequency until death occurred. In general, the respiratory movements following injection of stingray venom were almost always of a different nature than those seen before the injection. They tended to be more superficial, more irregular, and more abdominal in type. Occasional gasping movements were not uncommon.

Unfortunately, as only lethal doses of weeverfish venom were used in the present experiments, no data are available on the minimal changes that might be produced by this toxin. Lethal amounts of the venom produced apnea of 10- to 40-sec. duration, followed by irregular and often gasping respirations.

Maintenance of the respiratory cycle with induced artificial respiration did not appear to influence the initial cardiovascular depression.

In several animals the chest was opened immediately following clinical death (apnea and absence of pulse); the heart was still found to be contracting. This has frequently been seen following injection of lethal doses of stingray venom, and has been reported following administration of stonefish venom.[38] This should not be interpreted to mean that the heart is maintaining either effective contractions with an adequate stroke output, or normal electrical activity. It has been demonstrated that the cardiac output is markedly depressed following injection of lethal doses of stingray and weeverfish venoms, and that the focus for the contractions is probably elaborated outside the sinoauricular node. These changes appear to be irreversible.

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PHARMACOLOGICAL STUDY OF POISONS FROM SHELLFISH AND PUFFER FISH

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Paralytic shellfish intoxication has been a major public health problem in many countries, including the United States. In July 1927, 102 cases of mussel poisoning, with six deaths, occurred in California near Golden Gate (Meyer *et al.*, 1928). Clinical symptoms in these cases were primarily peripheral paralyses, and varied from tingling and numbness around the lips, gum and tongue, finger tips, and toes to loss of strength in the muscles of the neck and extremities, dyspnea and death by respiratory failure. Two recent nonfatal cases of mussel poisoning occurred a short distance north of the Golden Gate (Seven, 1958). McFarren *et al.* (1957) have listed 602 cases of such poisonings throughout the world, with 69 deaths, from 1798 to 1954.

Many of the samples of mussel poison used in prior investigations were of varying purity and potency. Although the effects of paralytic shellfish poison have been described previously (Meyer *et al.*, 1928; Prinzmetal *et al.*, 1932; Kellaway, 1935; Hashimoto and Migita, 1950; Sapeika, 1953; Fingerman *et al.*, 1953; Bolton *et al.*, 1959), the accessibility of highly purified samples of mussel poison (from *Mytilus californianus*) led us to repeat some of the earlier observations and to undertake some additional studies. The availability, also, of samples of poison from Alaska butter clams (*Saxidomas giganteus*) permitted us to compare the effects caused by this material with those produced by the mussel poison.

Another important cause of food poisoning is the ingestion of toxic puffer fish. This problem is especially prevalent in Japan, where these fish are a major food item. Such poisoning is rare in the United States but two fatal cases have occurred in Florida (Benson, 1956). Japanese investigators (Iwakawa and Kimura 1922; Yano, 1938) have reported that puffer poison causes a curarelike effect, depresses conductivity in motor and sensory nerves and in sympathetic fibers, and depresses medullary centers. Other studies of puffer poison have been published recently (Kuriaki *et al.*, 1956; Kuriaki and Wada, 1957; Schramm, 1957; Murtha *et al.*, 1957; Tsuda *et al.*, 1958; Furukawa *et al.* 1959). Again, many of the prior investigators worked with puffer poison samples of varying purity and potency. The accessibility of a highly purified crystalline hydrochloride of the poison permitted us to repeat some of the earlier observations and to undertake some additional studies.

*Materials**

The mussel and clam poisons used in these experiments were extracted and purified by methods described by Schantz *et al.* (1957) and contained 5000 to 6000 mouse units (MU) per mg. of solids. Evidence for the purity of this material has been presented by Mold *et al.* (1957). The intravenous, certainly-lethal dose of this mussel poison is 3 to 4 μg./kg. (15 to 20 mouse units/kg.). The clam poison samples used, although less concentrated, had about the same toxicity.

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* The shellfish poison samples were supplied by Edward J. Schantz, Chemical Corps Biological Laboratories, Ft. Detrick, Md.

The crystalline hydrochloride of puffer poison has an I.P. LD_{50} for mice of about 0.02 μg./gm., killing the mice within 3.4 to 3.7 min. after injection.

Both anesthetized and unanesthetized cats, dogs, and rabbits of mixed breed and sex were used in these experiments.

Results

FIGURE 1 shows the effect of an intravenous injection of 15 MU/kg. of mussel poison into the anesthetized cat. Mussel poison has a marked effect on both respiration and blood pressure. As may be noted in a later illustration concerned with our attempts to reverse these effects (FIGURE 7), an I.V. injection of 20 MU/kg. usually causes a more precipitous fall in blood pressure.

Cardiovascular effects. While studying in these laboratories the effect of mussel poison on the isolated rabbit heart prepared by the Langendorff method (1895), Roe E. Wells (unpublished observations) found that perfusion of the heart at rates of 15 to 20 MU/min. soon stopped the heart permanently. When smaller concentrations were tested, decreases in rate and contractility occurred, but they were reversed rapidly when the poison infusion was stopped.

Immediately following intravenous injections of lethal doses of mussel poison, electrocardiographic records showed bradycardia and bundle-branch block. High doses of the poison (3 LD_{50}) usually produced AV dissociation. If artificial respiration and epinephrine were given prior to death, the ECG record quickly reverted to a more normal pattern; a prolonged PR interval and an elevated or depressed S-T segment often persisted, however.

The typical effects of mussel poison on the isolated cat papillary muscle are illustrated in FIGURE 2. In this experiment the poison produced a prompt, reversible depression of cardiac muscle contractility. A concentration of 40 MU in 75 ml. of Ringer-Locke's solution produced an immediate inability of the muscle to contract during electrical stimulation. After removal of the poison and several washings with fresh Ringer-Locke's solution, partial recovery occurred within 2 min. and complete recovery occurred within approximately 10 min. Similar effects occurred in a papillary muscle that continued to contract spontaneously and rhythmically following an initial brief electrical stimulation. Mussel poison (25 MU in 75 ml. of Ringer-Locke's solution) immediately abolished spontaneous contractions; following washings the spontaneous activity returned in about 2 hours.

When spontaneous contractions were abolished immediately following poisoning, the muscle still contracted in response to electrical stimulation, but its contraction height was reduced to approximately 50 per cent of the prepoisoning level. Small concentrations (5 MU in 75 ml. of Ringer-Locke's solution) of mussel poison usually produced a slight decrease of the papillary muscle's contraction height during electrical stimulation; this depression was rapidly reversed with washing.

Following an intravenous injection of mussel poison (20 MU/kg.), cardiac force in the anesthetized, bilaterally vagotomized dog decreased about 50 per

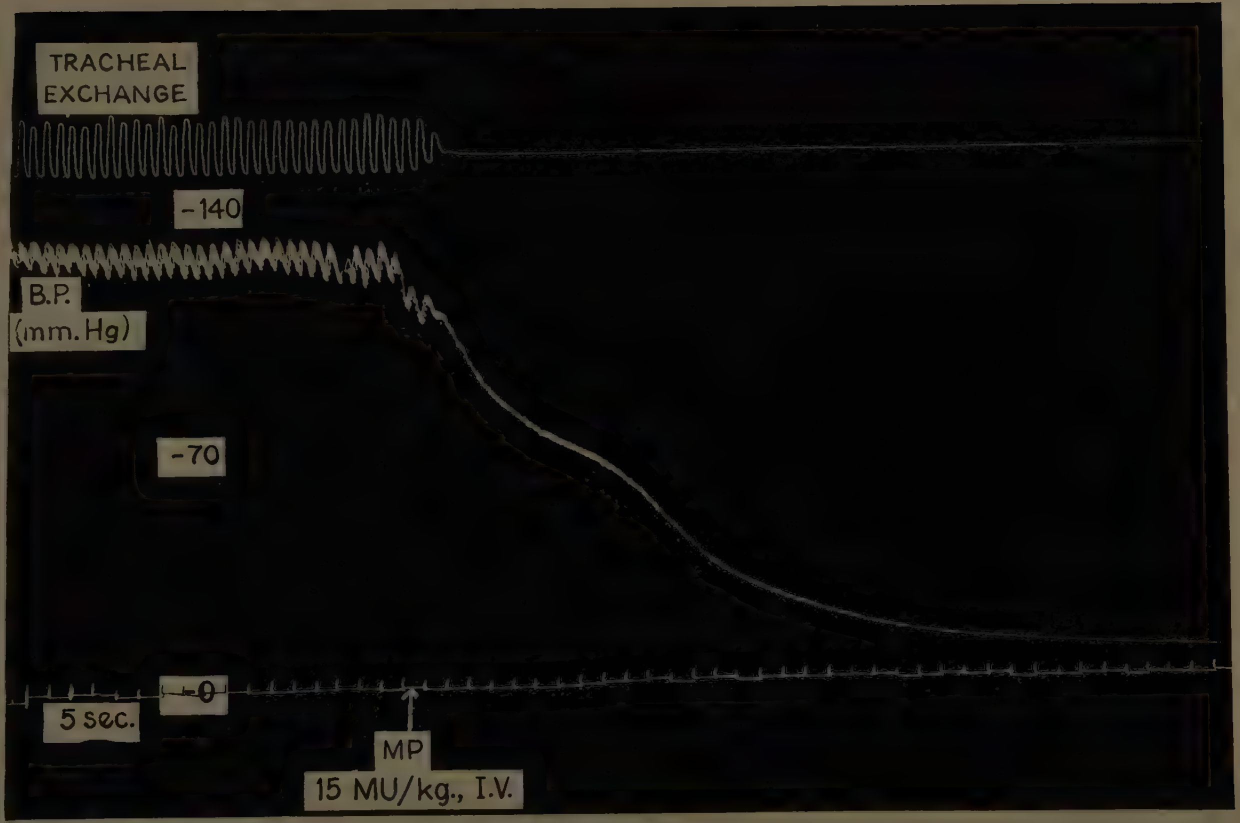


FIGURE 1. The effect of mussel poison on the anesthetized cat. Upper trace is respiration, middle trace is blood pressure (in mm. Hg), and lower trace is the time marker (each gradation = 5 sec.). Mussel poison (MP) was injected intravenously at the arrow, in a dose of 15 MU/kg.

cent within about 1 min. and to less than 10 per cent of the prepoisoning level within about 13 min. There was simultaneously a precipitous decrease in blood pressure. A partial recovery of heart contractile force and blood pressure occurred gradually during the next 2 hours.

The fall in blood pressure observed in partially eviscerated cats, in cats with the aorta and vena cava tied off just below the diaphragm, and in cats with the circulations of the fore limbs interrupted just outside the thoracic cage, was essentially as precipitous as that observed following poisoning of intact cats. Experiments involving synchronous records of arterial and venous pressures showed that the prompt fall in blood pressure apparently preceded any change in venous pressure. There was no evidence of decreased venous return before cardiovascular collapse. Mussel poison did not produce vasodilatation in the plethysmograph-enclosed leg or in the oncometer-enclosed kidney of the cat. Mussel poison did not affect the rate of flow through the vascular system of the isolated rabbit ear. The cardiovascular collapse produced by the poison

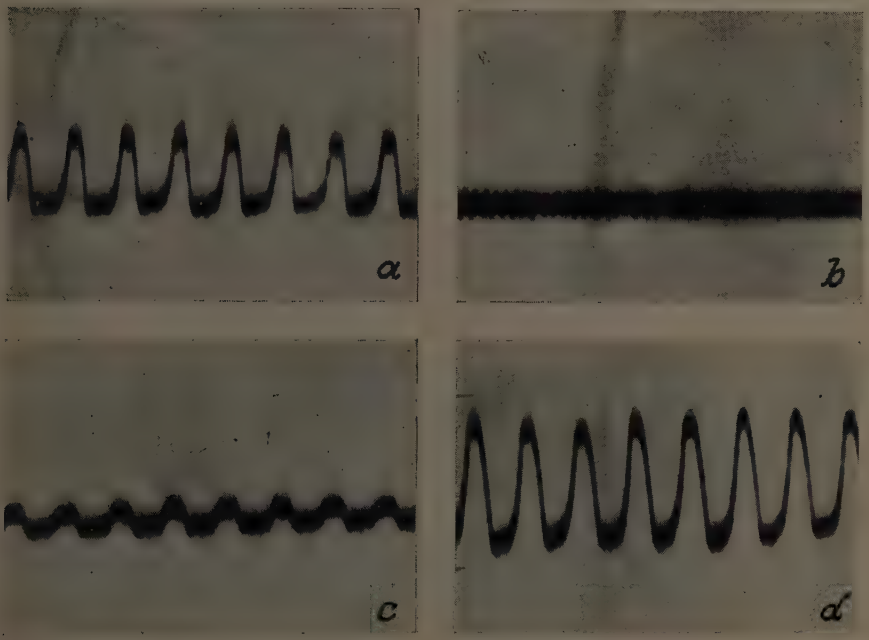


FIGURE 2. The effect of mussel poison on isolated cat papillary muscle. Photograph *a* shows contractions immediately before poisoning (during stimulation). Photograph *b* shows contractions about 1 min. after adding mussel poison to the bath (40 MU in 75 ml. of Ringer-Locke's solution). Small vertical lines are mechanical artefacts caused by bubbling oxygen-carbon dioxide gas through the solution. The mussel poison was removed from the bath immediately after photograph *b* was made, and the papillary muscle was washed several times with fresh Ringer-Locke's solution. Photograph *c* was taken about 2 min. after the washings. Photograph *d* shows contractions about 10 min. after the washings. At this time the contractions were slightly greater than those recorded before poisoning, but they soon returned to control levels.

in bilaterally vagotomized cats was essentially the same as that produced in intact cats.

The cardiovascular effects of mussel poison in an artificially ventilated cat with a high cervical cord section and bilateral vagotomy are illustrated in FIGURE 3; two factors are apparent in this figure: the prepoisoning blood pressure is low and the drop in blood pressure after administration of mussel poison is slight and gradual. This figure is typical of eight similar experiments. To investigate the possibility that the comparatively slight response to injection of mussel poison depended upon the low initial level of blood pressure in the cord-sectioned cats, five intact, anesthetized, artificially-ventilated cats were bled until their blood pressures approximated those seen in the cord-sectioned animals. A typical response by one of these cats to intravenous injection of 20 MU/kg. of mussel poison is shown in FIGURE 4. The drop in blood pressure was more marked and more sudden than that seen in the cord-sectioned cats. Repeated intravenous injections of sublethal and lethal doses of mussel poison usually had very little effect on the blood pressure of cervical cord-sectioned, vagotomized cats, but usually produced marked decreases in blood pressure in the bled, intact cats. One experiment was performed on a cat with high cervical cord section and intact vagi; following the intravenous injection of a lethal dose of mussel poison in this cat there was only a slight, slow fall in blood pressure.

Effects on the phrenic nerve and diaphragm. Following I.V. doses greater than 10 MU/kg. of mussel poison in the artificially respired cat, the height of all phrenic nerve spike potentials was reduced approximately 40 to 60 per cent, and electrical stimulation of the phrenic nerve did not produce contraction of the diaphragm. A reduced contraction often occurred when the diaphragm was stimulated directly with voltages of high intensity (about 100 v). Smaller doses (less than 10 MU/kg.) of the poison caused slight reductions in the height of both nerve spike potentials and the diaphragm's contractions.

Effects on reflexes. FIGURE 5 illustrates the effects of mussel poison on the knee-jerk reflex in the anesthetized, artificially respired cat. Doses of 10 MU/kg. of the poison rapidly produced a complete failure of the quadriceps muscle to contract following reflex stimulation at a time when stimulation of the muscle's motor nerve produced muscular contraction. Recovery from the effects on nerve and reflex transmission in this preparation usually occurred simultaneously.

In another reflex study with the anterior tibialis muscle preparation in the anesthetized, artificially respired cat, I.V. injection of 10 MU/kg. of mussel poison completely abolished contractions of the muscle during a slow rate (30/min.) of electrical stimulation of the reflex pathway; the contractions produced by a fast rate (50/sec.) of stimulation were reduced more than 50 per cent. At this time stimulation of the muscle's motor nerve produced a contraction that was essentially normal. Over a period of 10 to 20 min., however, the latter contraction height also decreased. Larger doses (15 to 20 MU/kg.) of the poison produced simultaneous paralysis of reflex and peripheral transmission.

In these experiments, nerve spike potential height recorded from the muscle's motor nerve was reduced approximately 40 to 60 per cent during intoxication.

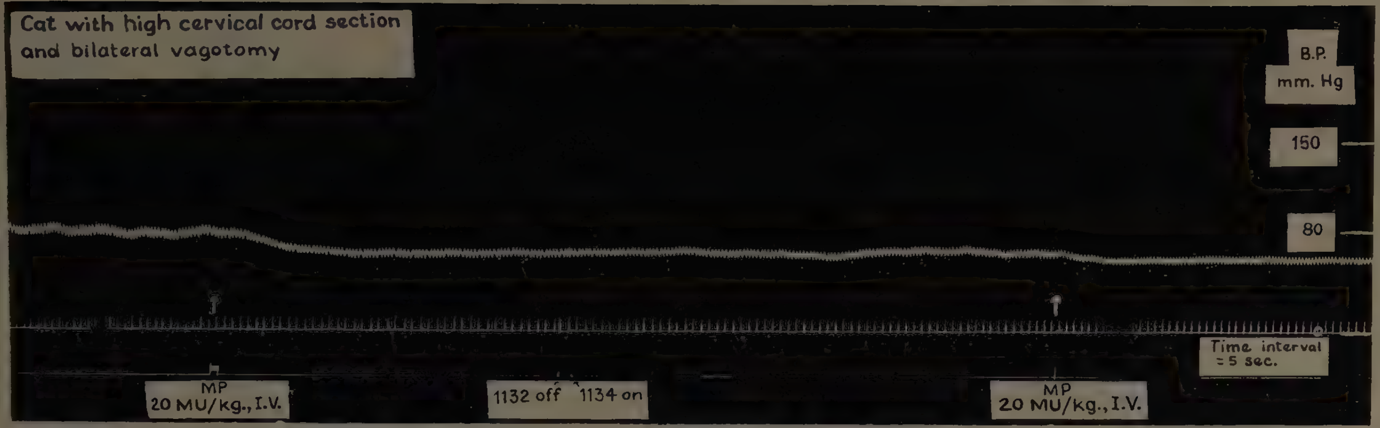


FIGURE 3. The cardiovascular effect of mussel poison on a cervical cord-sectioned, bilaterally vagotomized cat. The animal was respired artificially. Blood pressure (BP) is indicated in mm. Hg; each timer mark = 5 sec. Mussel poison (20 MU/kg.) was injected intravenously at signal marks MP (*arrows*).

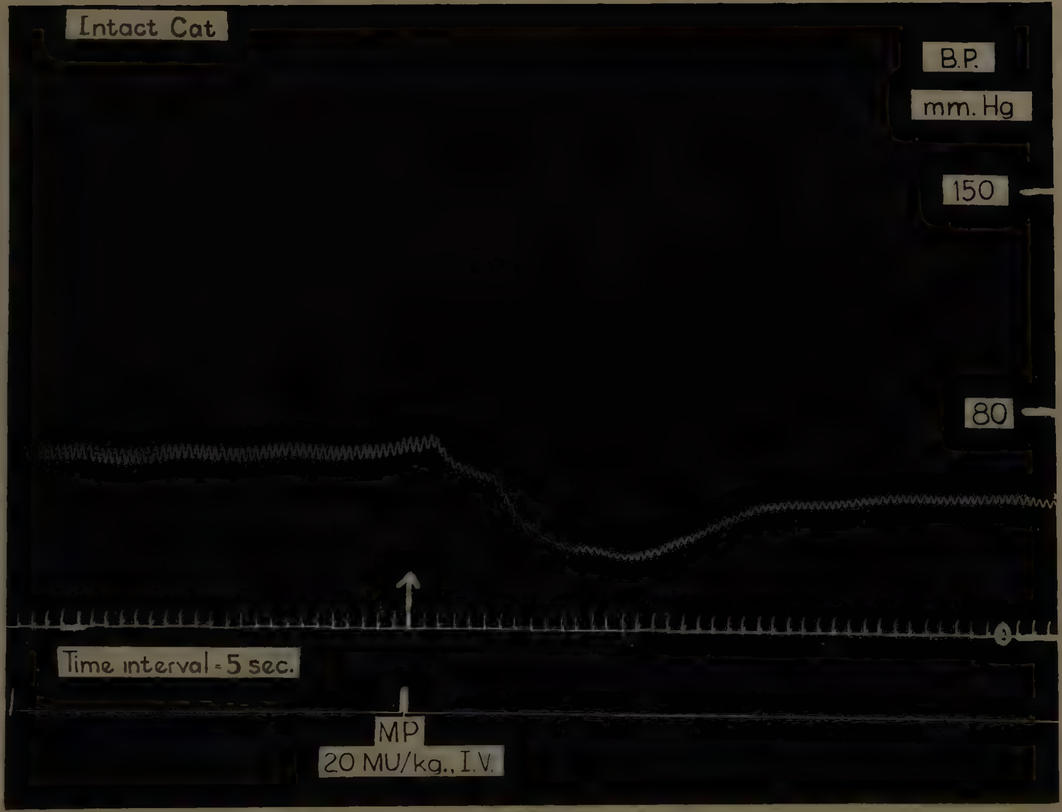


FIGURE 4. The cardiovascular effect of mussel poison in the intact, artificially-respired cat with low blood pressure. Blood pressure was reduced to about 70 to 75 mm. Hg by partial exsanguination approximately 45 min. before poisoning. Blood pressure (BP) is indicated in mm. Hg; each timer mark = 5 sec. Mussel poison (20 MU/kg.) was injected intravenously at signal mark MP (*arrow*).

The depression of reflex and peripheral transmission in this preparation was reversed in recovery; the recovery from the effect on peripheral transmission occurred more promptly than recovery from the effects on reflex transmission. In some experiments, decreases in peripheral nerve or reflex transmission were observed when blood pressure levels were subnormal, but in other experiments these same effects occurred when blood pressure was essentially normal. Recovery from these effects was observed frequently when blood pressure levels were subnormal.

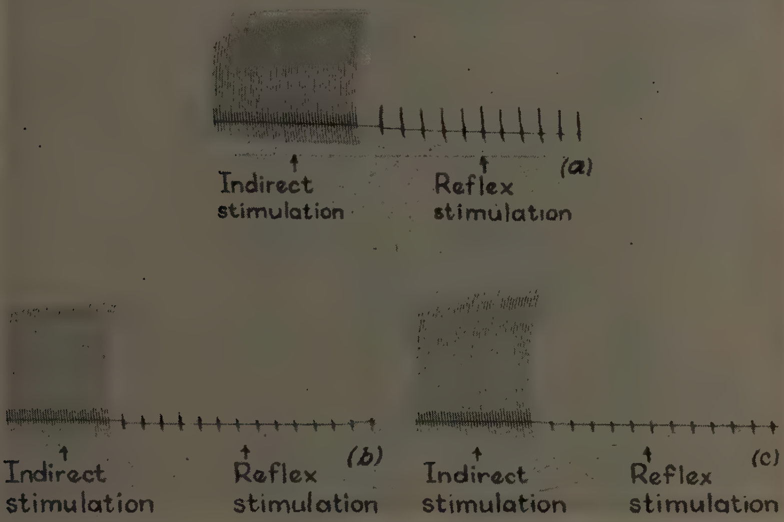


FIGURE 5. The effect of mussel poison on the knee-jerk reflex. The anesthetized cat was respired artificially throughout the experiment. The record of mechanical activity represents movement of the ankle in response either to motor nerve (anterior crural nerve) stimulation (intensity = 0.3 v; duration = 0.1 msec.; frequency = 30/min.), marked Indirect Stimulation, or to blows delivered to the patellar ligament every 15 sec., marked Reflex Stimulation. (*a*) The response immediately before poisoning; (*b*) the response approximately 2 min. after intravenous (vena cephalica) injection of 10 MU/kg. of mussel poison; (*c*) the response about 10 min. after poisoning. The latter tracing of response to reflex stimulation was identical to that of the dead animal, and illustrates the slight mechanical artifact produced by taps on the patellar ligament.

The effect of mussel poison on the Lovén reflex is illustrated in FIGURE 6. Doses as low as 2 to 3 MU/kg. of the poison produced a marked reversible depression of this reflex. Recovery usually occurred within 3 to 20 min. after small doses, but the recovery period was longer after larger doses of the poison. The depression of the Lovén reflex occurred when the animals were well ventilated (respired artificially) and when blood pressure was at, or near, prepoisoning levels.

Clam poison. The pharmacology of clam poison appears to be identical to that of mussel poison, lethal doses causing prompt respiratory arrest and cardiovascular collapse. The effects of clam poison on reflex and neuromus-

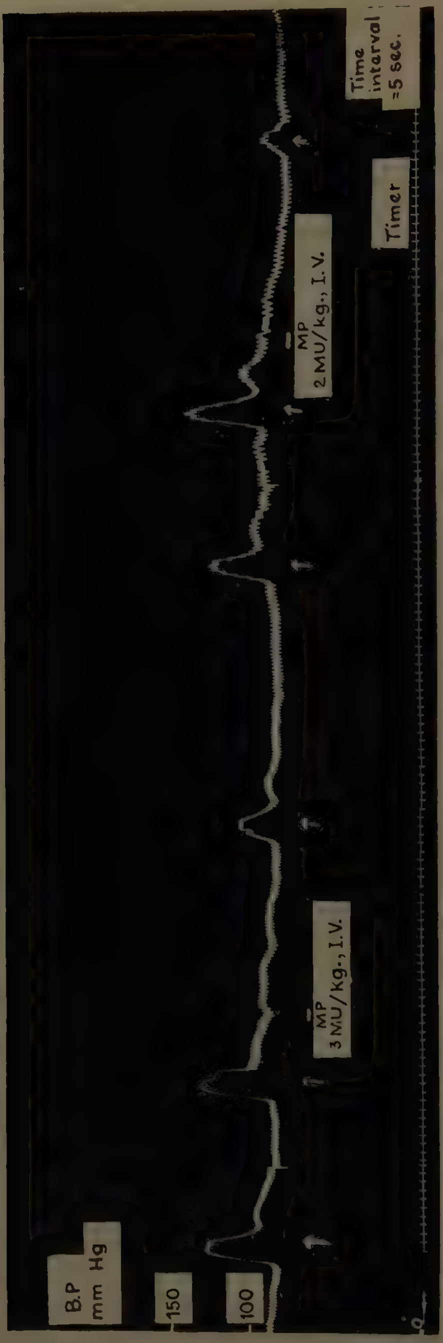


FIGURE 6. The effect of mussel poison on the Lovén reflex. The anesthetized cat was respired artificially throughout the experiment. Blood pressure (BP) is indicated in mm. Hg; each timer mark = 5 sec. Arrows show blood pressure during supramaximal stimulation of sciatic nerve (intensity = 3 v; duration = 1 msec.; frequency = about 150/sec.). Mussel poison was injected intravenously (vena cephalica) at the marks MP; the first dose was 3 MU/kg. and the second was 2 MU/kg.

cular transmissions were identical to those of mussel poison. Earlier, less pure samples of clam poison appeared to be somewhat different from mussel poison. The crude samples, for example, contained a potent factor producing emesis even in anesthetized animals. This factor disappeared during purification.

Treatment of shellfish intoxication. Artificial respiration was the most effective single measure for treating the effects produced in cats by intravenous lethal doses of shellfish poison. Several anticurare drugs (such as neostigmine) were tested as adjuncts to artificial ventilation, but were found to be ineffective. DL-Amphetamine (Benzedrine), epinephrine, ephedrine, and DMPP (1,1-dimethyl-4-phenylpiperazinium iodide) were valuable adjuncts to artificial respiration when the animals were poisoned with 15 MU/kg. I.V. of mussel or clam poison, followed within 5 to 15 min. by another 5 MU/kg. I.V. DL-Amphetamine appeared to be particularly effective.

With reference to recovery time only, none of these drugs added significant value to artificial respiration in treating the effects of a single intravenous dose of 20 MU/kg. of mussel poison. In a single experiment, however, DL-amphetamine resulted in complete recovery from the effects of this dose of poison in approximately 10 min. (FIGURE 7). The recovery time for cats poisoned intravenously with 20 MU/kg. of mussel poison and treated with artificial respiration alone was usually 30 to 45 min., some recoveries requiring considerably longer (90 or more min.).

Puffer poison. In unanesthetized cats, puffer poison in doses of 1 to 200 μg./kg., depending on the route of administration, caused retching and vomiting, ataxia, dyspnea, and paralysis of the limbs. The hind limbs were particularly sensitive to the poison but all the limbs were affected with higher doses or at a later time. An ascending type of paralysis was observed. The I.V. and I.P. LD_{50}s for the cat appeared to be less than 10 μg./kg. A dose of 200 μg./kg., however, when given by mouth to a cat did not cause death.

FIGURE 8 shows the effect of puffer poison on respiration and blood pressure. Intravenous injections of 5 μg./kg. of the poison usually caused rapid cessation of respiration and a precipitous fall in blood pressure; in some experiments these effects occurred more slowly. Animals with bilateral vagotomy and transection of the cervical cord showed a hypotension similar to that of intact cats with blood pressure levels lowered to the same level as that of the first group. There was no significant effect on gut activity other than a slight inhibition in a few animals.

A reduced voltage of the electrocorticogram occurred following I.V. injections of 5 to 7 μg./kg. of the poison, but this appeared to be related to the blood pressure depression.

No significant abnormalities of the ECG, other than a decreased rate, usually occurred in cats poisoned with 3 to 6 μg./kg., I.V., of the poison. In one experiment, however, a partial AV block occurred for several minutes and, in another cat, auricular activity was abolished a few minutes before death. The absence of major ECG abnormalities was also observed in the anesthetized, bilaterally vagotomized, artificially ventilated (open chest) dog following I.V. injections of 5 μg./kg. At the same time, however, a rapid fall in blood pres-

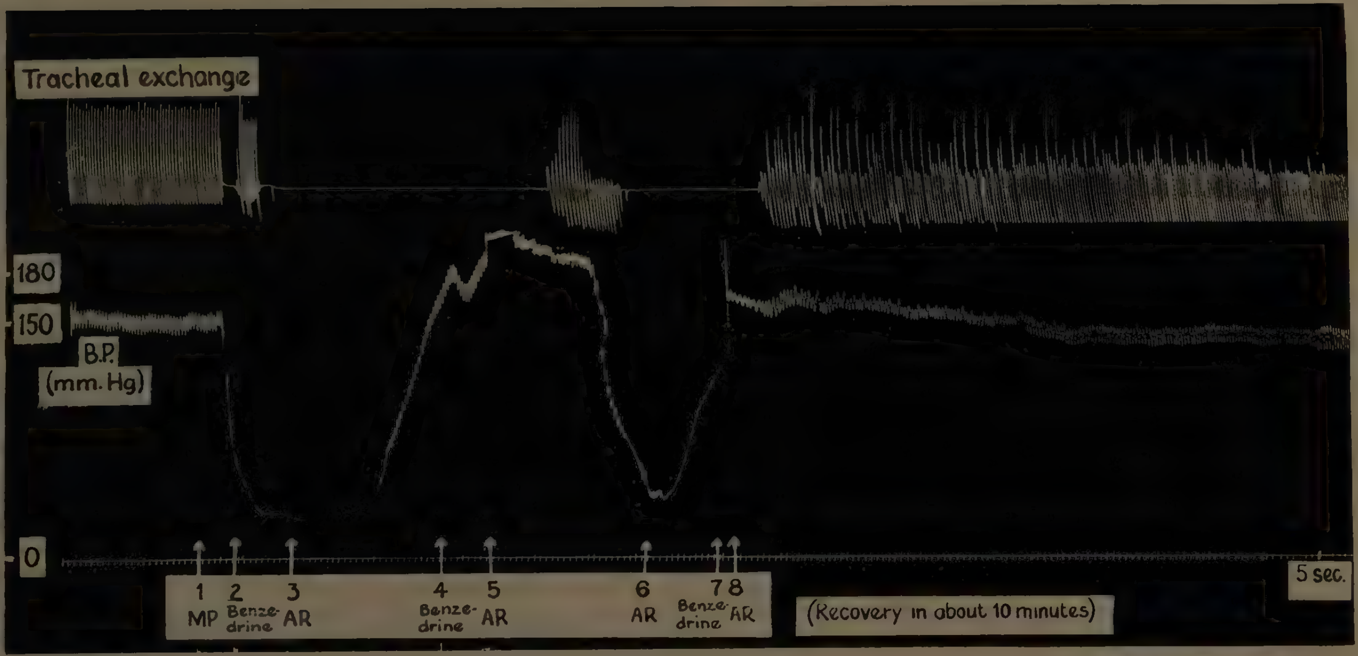


FIGURE 7. The effect of DL-amphetamine (Benzedrine) as an adjunct to artificial respiration in the treatment of mussel poison intoxication in the cat. The upper trace is respiration; the center trace is blood pressure (BP) in mm. Hg; each timer mark in the lower trace = 5 sec. Mussel poison (MP), 20 MU/kg., was injected intravenously at *arrow* 1 (time 1005 hours). DL-Amphetamine (Benzedrine) was injected intravenously at *arrow* 2 (2 mg./kg.), *arrow* 4 (1 mg./kg.), and *arrow* 7 (0.5 mg./kg.). Artificial respiration (AR) was begun at *arrow* 3, turned off temporarily at *arrow* 5, started again at *arrow* 6, and stopped permanently at *arrow* 8 (1015 hr.).

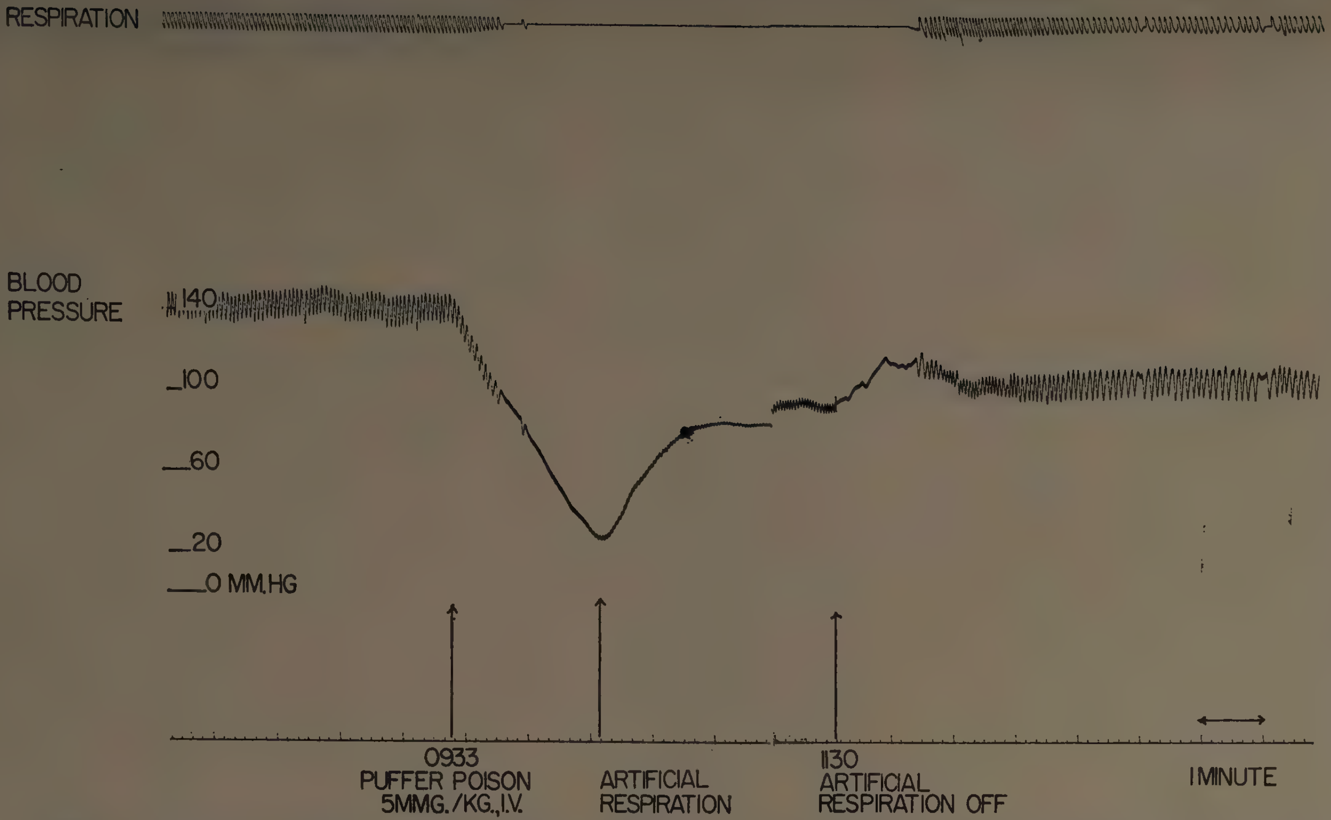


FIGURE 8. The effect of puffer poison on respiration (upper trace) and blood pressure (lower trace) of the cat. The animal was injected intravenously with 5 μg./kg. of the poison at the first *arrow* (0933 hr.). Interrupted positive pressure air was used for artificial ventilation. Reproduced by permission of *The Journal of Pharmacology and Experimental Therapeutics*, **122:** 247.

sure and a marked decrease in contractile force of the heart occurred (FIGURE 9).

In several cat experiments intracarotid injections of puffer poison (5 μg./kg.) caused a greater depression of spontaneous respiration than that which occurred following intravenous injections.

Perfusion of the isolated rabbit heart for 80 min. with a concentration of 3.3 μg. of puffer poison/100 ml. had no significant effect. The isolated rabbit gut was not affected by concentrations of 13.3 μg./100 ml.

Close intra-arterial or intravenous injections of 4 to 10 μg./kg. of the poison greatly affected neuromuscular function. In the 4 μg./kg. dose range, the gastrocnemius-soleus muscle group's response to slow rates (30/min.) of supramaximal electrical stimulation of the motor nerve was inhibited rapidly. Later, the response to more rapidly repetitive (30/sec.) motor nerve stimulation was inhibited. Following 10 μg./kg., a complete block of neuromuscular transmission occurred rapidly. A gradual inhibition of the responsiveness of skeletal muscle also occurred so that the muscle failed to respond to direct, supramaximal stimulation when neuromuscular transmission was blocked. During recovery, the direct effect on the muscle was reversed first, followed by a return of contractions during repetitive motor nerve excitation and, finally, a return of contractions during the slow rate of motor nerve excitation.

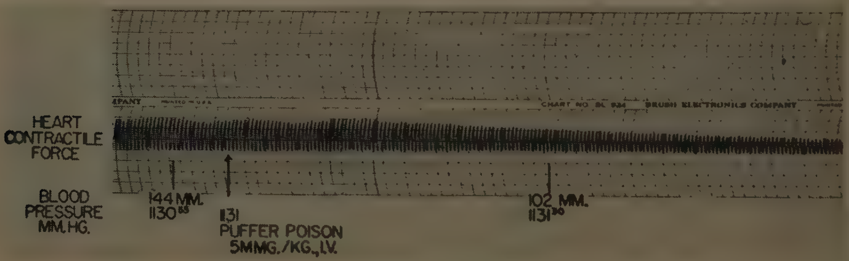


FIGURE 9. The effect of puffer poison on the contractile force of the dog's heart. The animal was anesthetized with sodium pentobarbital (30 mg./kg. I.V.), bilaterally vagotomized,

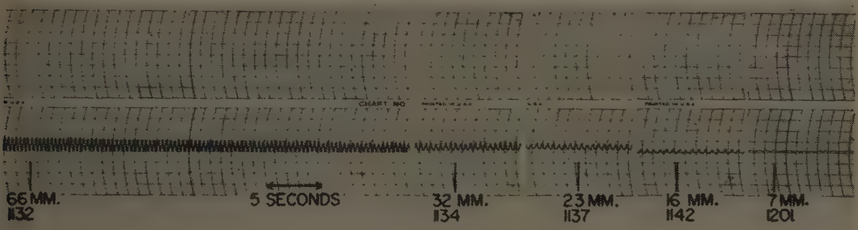
In another series of similar experiments in anesthetized cats, an ascending type of paralysis was observed, affecting the hind limbs initially, then the diaphragm, and finally the fore limbs. This effect was demonstrable only within a limited dose range (5 to 6 μg./kg., I.V.). When a larger dose (10 μg./kg., I.V.) of the poison was injected, neuromuscular paralysis occurred very rapidly and apparently simultaneously in both the diaphragm and hind limbs, but the fore limbs were not paralyzed until several minutes later. The diaphragm was found to be unresponsive to direct, electrical stimulation but this effect was reversed later, the diaphragm then responding to direct stimulation, although direct stimulation of the hind limb muscles did not produce contraction.

Treatment of puffer poisoning. As with shellfish intoxication, artificial respiration is the most important therapeutic measure during puffer poisoning.

We have concluded that pentylenetetrazol (Metrazol) is a valuable adjunct to artificial respiration and shortens recovery time below that obtained with artificial respiration alone. In one experiment, for example, where Metrazol was tested as an adjunct to artificial respiration in the poisoned cat, an improved blood pressure and restoration of spontaneous respiration occurred within about 18 min. after artificial respiration was administered. The usual recovery times for cats poisoned with this dose range (5 to 8 μg./kg., I.V.) and treated with continuous artificial ventilation alone were from 1.3 to 5.3 hr.

Discussion

Both central and peripheral effects seem to be involved in the respiratory arrest and cardiovascular collapse caused by lethal doses of mussel and clam poisons. Concerning the effect on respiration, it has been reported recently (D'Aguanno, 1959) that shellfish poison depressed respiration centrally with no curarization peripherally.



and ventilated artificially with interrupted positive pressure (room air). Reproduced by permission of *The Journal of Pharmacology and Experimental Therapeutics*, **122:** 247.

In investigating the cardiovascular effects of the poison, the electrocardiographic studies showed that mussel poison interfered with myocardial conduction and this effect was associated with cardiovascular collapse. Heart contractile force also was reduced following intoxication.

The direct depressant effect of mussel poison on the myocardium has been reported previously (Sapeika, 1953). The effects of mussel poison on the isolated rabbit heart, as shown by Wells (unpublished observations), demonstrated that the poison apparently is quickly washed out of cardiac muscle. This observation was confirmed in the papillary muscle studies, where it was noted that the poison apparently was not readily fixed permanently to cardiac muscle within the dose range tested. The latter experiments demonstrated that mussel poison produces a direct depression of the myocardium. The direct depression of the myocardium, although shown to be readily reversed with washings in the papillary muscle studies, is probably an important factor contributing, in part, to the immediate cardiovascular collapse produced by lethal doses of mussel poison.

Kellaway (1935) has shown that his sample of mussel poison caused vasodilatation in the splanchnic region whereas Sapeika (1953), on the other hand, found that his sample of poison did not produce dilatation of intestinal vessels. The results of our experiments on the isolated perfused rabbit ear, on eviscerated cats, on cats with occluded circulation, on cats with an oncometer-enclosed kidney, on cats with a plethysmograph-enclosed leg, and on cats from which synchronous arterial and venous pressure were recorded, have shown that this sample of poison apparently has no major vascular action. Peripheral vasodilatation, therefore, apparently is not an important factor contributing to the marked cardiovascular collapse produced by our sample of mussel poison. The cardiovascular collapse probably is not caused primarily by hyperactivity of the cardio-inhibitory branches of the vagi, since the effects that occur following the poisoning of bilaterally vagotomized cats are essentially the same as those noted in intact cats.

Results obtained from studying the cardiovascular effects of mussel poison in cervical cord-sectioned, bilaterally vagotomized cats show that the rapid fall in blood pressure following lethal doses of mussel poison is produced, in part, by the actions of the poison on the brain. The one experiment where the cervical cord was sectioned and the vagi were intact suggests that this effect may be mediated through the spinal cord. The precipitous fall in blood pressure that occurs immediately after intravenous injection of lethal doses of mussel poison in intact cats does not occur usually in the cervical cord-sectioned, bilaterally vagotomized cats. However, small decreases in blood pressure occurred usually in the latter animal, suggesting that the cardiovascular collapse was not caused entirely by the central actions of the poison. Again, the effects of additional doses of mussel poison were quite different in the cord-sectioned, bilaterally vagotomized cats and the intact animals. As illustrated in FIGURE 3, a second intravenous injection of 20 MU/kg. of mussel poison, administered approximately 12 min. after the initial 20 MU/kg. dose, apparently had very little effect on the blood pressure of the cervical cord-sectioned, bilaterally vagotomized cat. In intact cats, additional doses of the poison usually produced additional decreases in blood pressure.

The various cardiovascular studies have shown that the immediate cardiovascular effects that occur in mussel poisoning are produced, in part, by the actions of mussel poison on the brain and, in part, by a direct depression of the myocardium.

This sample of poison can readily affect the height of induced phrenic nerve spike potentials and the ability of the intact cat's diaphragm to contract during electrical stimulation.

Our reflex studies again demonstrated that mussel poison is a very potent neurotoxin, capable of producing complete depression of both peripheral nerve and reflex transmission. The effect of the poison on reflex transmission is apparently greater than that on peripheral nerve, since attempts to elicit muscle contraction by stimulation through the reflex pathway were completely ineffective at times when stimulation of the muscle's motor nerve elicited excellent contractions. The results of the anterior-tibialis studies showed that recovery from the effects of the poison on peripheral nerve usually occurred more promptly than recovery from the effects on a reflex arc. In the knee-jerk reflex, however, the recovery was usually simultaneous. Our data indicate that the neurotoxic effects of mussel poison probably are not due to impaired circulation arising from low systemic blood pressure, since decreases in nerve transmission sometimes occurred when blood pressure levels were at, or near, normal levels, and recovery sometimes occurred when blood pressure levels were subnormal.

Depression of the Lovén reflex was also shown to be apparently independent of significant effects on systemic blood pressure levels. Mussel poison produced a reversible depression of this reflex when blood pressure levels were at, or near, normal levels and the animals were being well ventilated. The effects of mussel poison on the Lovén reflex could be on the afferent or efferent pathways, on the higher centers, or on a combination of these sites. The exact site or sites of this action has not been determined in this investigation, but these results suggest that the poison may affect the cardiovascular regulating mechanism.

The studies concerned with a comparison of the pharmacological effects caused by clam poison with those caused by mussel poison strongly suggest that the two toxic factors may be very similar in structure, if not identical. This conclusion is in agreement with a recent similar one (Mold, 1957) based on chemical studies of these poisons.

Like shellfish poison, puffer poison is quite toxic. Some differences have been observed between the effects of puffer poison and those of shellfish poison. Shellfish poison is more toxic than puffer poison in the dog and cat. Within the dose range tested, puffer poison usually had only a slight effect on the ECG of the dog and cat, and no apparent effect on the isolated rabbit heart, whereas shellfish poison caused marked cardiotoxic effects.

Our observations concerning the effects of the crystalline puffer poison substantiate many of the conclusions of Japanese investigators (Iwakawa and Kimura, 1922; Yano, 1938). Like them, we find that comparatively low doses of the poison can readily inhibit neuromuscular function. Other major effects include respiratory failure and hypotension. Our observation that an intracarotid injection of this poison causes a more rapid inhibition of spontaneous respiration than an intravenous injection is highly suggestive that the poison has a direct action on the respiratory centers.

The retching and vomiting that occurred in unanesthetized cats during puffer intoxication were never seen in anesthetized cats or dogs. Similarly, puffer poison caused very little, if any, effect on gut activity *in vivo* in anesthetized cats and no apparent effect *in vitro*. These findings suggest that the retching and vomiting induced by puffer poison are elicited by action of the compound on the chemoreceptive trigger zone of the area postrema.

Although puffer poison had no apparent major, direct cardiotoxic effects within the dose range tested, the substance still caused significant decreases in cardiac contractile force associated with hypotension. It has been reported that this poison depresses the vasomotor centers (Iwakawa and Kimura, 1922; Yano, 1938). This finding and the findings (Iwakawa and Kimura, 1922; Yano, 1938; Nagayosi, 1941) that blockage of peripheral sympathetic fibers may contribute to the hypotension would suggest that the negative inotropic effect observed in the intact animal is caused almost entirely by indirect actions of puffer poison.

The neuromuscular paralysis may be due to inhibition of conductivity in nerve reported by other investigators (Iwakawa and Kimura, 1922; Katagi, 1927). We have not studied this aspect, other than finding that the poison can affect the response of skeletal muscle to both indirect and direct excitation.

It is interesting to note, also, that the neuromuscular apparatus of the hind limbs of the cat apparently is more sensitive to the action of puffer poison than that of the fore limbs or diaphragm. Although the observation has been reported previously (Yano, 1938), the ascending type of paralysis that occurs during puffer intoxication apparently has not received adequate recognition.

Summary

The shellfish (from toxic mussels and clams) poison and puffer poison samples studied in this investigation are quite toxic. Both central and peripheral effects seem to be concerned with the physiological alterations that occur during intoxication by either poison. The pharmacological effects of clam poison appear to be identical with those of mussel poison, whereas some differences have been observed between the effects of puffer poison and those of shellfish poison.

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ANALYSIS OF THE TOXIN PRODUCED BY *GONYAULAX CATENELLA* IN AXENIC CULTURE*

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Historically, fish kills and poisonous shellfish have been connected with discolored waters, the so-called "red tides,"[1] which contain high concentrations of algae. *Gonyaulax catenella*,[2] a motile photosynthetic marine dinoflagellate, has been blamed as a source of mussel poison by several investigators.[3,4]

G. catenella is found in the coastal waters off California where, during blooms, it ranges from 20 to 40 million cells/l. In such waters, filter-feeding shellfish ingest these dinoflagellates, thus becoming toxic, especially during summer algal blooms. Shellfish store and concentrate the toxin of *G. catenella* with no apparent ill effects to themselves. However, ingestion of such contaminated shellfish by man can be fatal. The poison is one of the few natural toxins that approach botulinum in toxicity. Blooms of *G. catenella*, although fatal to man through the shellfish food chain, have never been reported as the direct cause of fish kills.

Most work on the toxin produced by *G. catenella* has been done with purified toxin extracted from digestive glands of California mussels, *Mytilus californianus*,[5,6] or from siphons of Alaskan butter clams, *Saxidomus giganteus*. Extensive chemical and physical analysis has been performed on the toxin extracted from the above sources,[7,8] but the general structure of the molecule has not yet been defined.

Some work on nonaxenic cultures has been undertaken, but the erratic growth effect of bacteria on toxin production and stability, together with generally poor yields of toxin, greatly limits the usefulness of these studies. Although axenic cultures are subject to variation in toxin production, they proved much more controllable; they are essential in order to eliminate bacteria as a possible source of the poison.

Growth of Organism

G. catenella was cultured axenically in a chemically-defined medium starting with a bacterized culture supplied by D. Foster of the G. W. Hooper Foundation, University of California Medical Center, San Francisco, Calif. Fernbach flasks, containing one liter of medium, were inoculated from Erlenmeyer flasks containing 75 ml. of medium that, in turn, were inoculated from test tubes containing 10 ml. of medium.

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Cultures were inoculated and harvested at the end of the growth phase (between 12 and 16 days) to avoid leaching of toxin into the medium. During log growth phase the poison is chiefly in the cells; supernatant when injected into mice showed no detectable poison, and cellular extracts were potent.

In large Fernbach flasks, cell populations as high as 45 × 10^6 cells/l. were obtained, yielding a maximum of 160 mouse units (MU) per flask. By definition, one MU is that amount of poison which, when injected intraperitoneally into a 20-gm. mouse, causes death in 15 min. In practice, however, greater precision is obtained if concentrations of 1.4 to 1.9 MU/ml. are used. This gives a median death time of 5 to 7 min.[9] Harvesting of 12 Fernbach flasks yields an average of 1300 MU.

Separation of Cells from Supernatant

In attempts to separate *G. catenella* cells from the supernatant, decantation was employed as a preliminary procedure, followed by filtration through Whatman No. 1 paper. The deposited cells were scraped off the paper and frozen. Centrifugation with a Serval continuous-flow centrifuge, operating at 5000 RPM and a flow rate of 3 l./hour, yields 90 per cent recovery of intact cells. Both methods were equally efficient in separating the cells from the supernatant. However, since centrifugation was faster, it was the procedure most often used. In any procedure that separated the cells from the supernatant, lysing of cells resulted in unrecoverable large losses of toxicity to the medium.

Extraction of Toxin from Cells

The toxin is readily liberated from the cells by rupturing the cell membrane. The most efficient method for cellular extraction is acid hydrolysis at elevated temperatures. Packed cells, 3 to 4 ml. in volume, are mixed with 30 ml. of distilled water and 1 ml. of 1.0 *N* HCl, and boiled for one-half hour.

Completeness of extraction was tested by grinding previously-acid extracted cells in a Virtis blender with an equal volume of glass beads, and then boiling for one-half hour with 25.0 ml. of 1.5 *N* trichloroacetic acid. The trichloroacetic acid was eliminated with ether before bioassay. No additional poison was recovered, indicating the completeness of the initial acid extraction.

Concentration and Purification of Toxin

Stability. The toxin is acid-stable. It decomposes rapidly in the basic range. It may be stored for months in an acid solution without loss of toxicity. Acid (*p*H 3.0) solutions, when boiled 3 to 4 hours, appreciably lose toxicity. Therefore concentration, when necessary, was by evaporation under reduced pressure.

Solubility. Results of extractions with polar and nonpolar solvents show that the toxin is soluble in water, methanol and, to some extent, in ethanol, but insoluble in all lipoid solvents.

Distillation. The toxin is neither steam-distillable nor volatile, even at reduced pressure in acid or basic solution. Twenty-five ml. of a toxic sample containing approximately 150 MU were added to 125 ml. of 1.0 *N* HCl solution and steam distilled under reduced pressure. As the distillation progressed, concentrated NaOH was added dropwise to the mixture until it was alkaline. The distillate was collected in 2.0 *N* HCl solution. Bioassay of the distillate for toxin was negative.

Adsorbants. Ion-exchange separations were done with various adsorbants in an attempt to parallel the purification procedures of the shellfish toxin.[5,10,11] These procedures are good, but less satisfactory when dealing with samples of relatively low toxicity and high salt concentration. The concentrated toxin extracted from *G. catenella,* when treated with charcoal or carboxylic acid resins XE-64 or IRC-50,* ran parallel to purified toxin isolated from mussels.

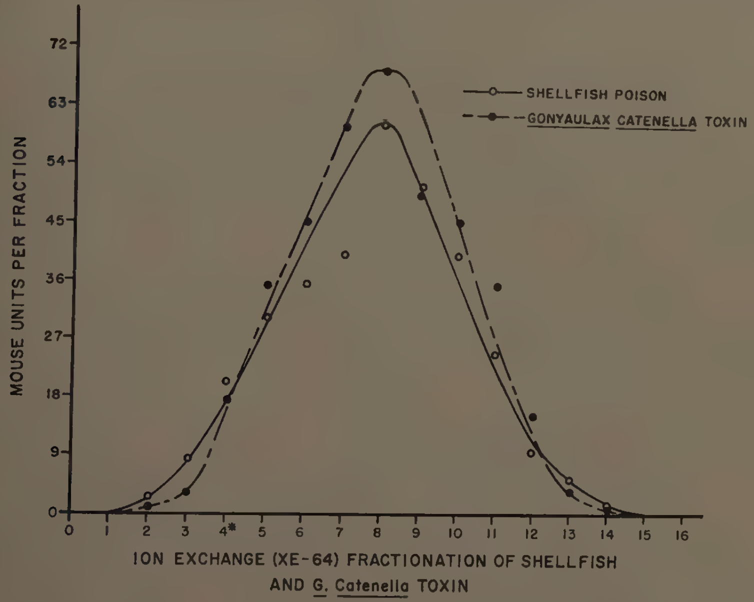

FIGURE 1. Comparison of the concentrations of *G. catenella* and shellfish toxin, eluted with 0.5 *N* trichloroacetic acid from an XE-64 resin column, and collected on a fraction-cutter. The 4th (*) fraction is that portion at which the *p*H fell below 4.5, as shown by Congo red indicator paper.

G. catenella toxin, a sample of shellfish toxin obtained from E. J. Schantz,[12] and a distilled water blank were run in parallel on columns of XE-64 resin adjusted to *p*H 5.2 at a flow rate of 30 drops/min. All columns were washed with additional buffer, eluted with 0.5 *N* trichloroacetic acid, and the eluate collected with an automatic fraction-cutter. Toxicity was located by assaying each collection tube. Both toxic samples were concentrated in those fractions collected after the *p*H had fallen below 4.5, as shown by Congo red indicator paper (FIGURE 1). Although recovery was satisfactory (about 70 per cent), a comparison of the total solids from all three columns showed an inherent solid concentration of approximately 20 mg./ml. Therefore samples of low toxicity did not yield toxin of sufficient purity (TABLE 1).

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* Rohm and Haas Company, Philadelphia, Pa.

Paper chromatography. The complicating factor in paper chromatography is interference by ionic contaminants that distort the retardation factors when sufficient toxin is used to obtain a positive Jaffé test.[13] When dealing with samples of low concentration, the use of a General Electric 75-w. Royal Blue beam bulb to scan the developed paper greatly intensified the orange color produced by the Jaffé reaction. Attempts to minimize the initial salt concentration by electric desalting and/or dialysis were unsuccessful; the toxin was dialyzable and recovery from the electro-desalter was poor. This necessitated the use of toxin samples of low concentrations in paper-chromatography experiments. The material used for most chromatography experiments was the toxin obtained from the XE-64 column, vacuum concentrated, spotted on strips of Whatman No. 1 paper, and run as an ascending chromatogram in a variety of solvents. Controls were shellfish toxin[5] (TABLE 2). The best solvent system was tetrahydrofurfural and water (17:3 v/v). Location of the toxin was determined by eluting 0.5-cm. paper sections with water, and then bioassaying.

TABLE 1

ION-EXCHANGE CHROMATOGRAPHY OF *G. CATENELLA* TOXIN AND SHELLFISH POISON ON XE-64 RESIN

Column number	*p*H	Material	Mouse units		Recovery (%)	Total solids of elutant	Total vol. of elutant
			Applied	Recovered			
1	5.35	*G. cat.**	500	325	65	22.2 mg./ml.	24.3 ml.
2	5.35	M.†	570	327	57	19.7 mg./ml.	24.3 ml.
3	5.30	H_2O	0	0	—	20.7 mg./ml.	24.4 ml.

* *G. catenella* toxin.
† Mussel poison.

TABLE 2

PAPER CHROMATOGRAPHY OF SHELLFISH POISON AND *G. CATENELLA* TOXIN

Solvent system	R_f values as indicated by bioassay	
	Shellfish poison	*G. catenella*
t-Butyl alcohol:acetic acid:H_2O (2:1:1)	0.28	0.32
t-Amyl alcohol:pyridine:H_2O (7:7:6)	0.49	0.51
Phenol:H_2O (4:1)	0.29	0.33
Tetrahydrofurfuryl alcohol:H_2O (17:3)	0.60	0.59

Electrochromatography. The migration characteristics of the toxin were investigated on paper-strip and curtain electrochromatography. A variety of buffers from *p*H 2.0 to 10.0 were studied. The best migration patterns were with $NaHCO_3$ buffer at *p*H 8.6 and a force field of 100 v. Purified mussel toxin moves in an electrical field and is detectable on the paper by the Jaffé reaction. *G. catenella* toxin did not move as fast in the electrical field under the same conditions, perhaps because of the high salt content or some other impurity present. When the same quantity of salt was added to the pure mussel toxin, or when pure mussel toxin and *G. catenella* toxin were mixed, the same flow pattern was obtained as in resin-extracted samples of the *G. catenella* toxin.

Adsorption curves. The resin-extracted toxin of *G. catenella*, treated according to the procedure developed by E. F. McFarren *et al.*[14] utilizing the Jaffé reagent, gave an adsorption curve similar to the mussel poison when measured on a Beckman Model B spectrophotometer.

Separation of toxin from the supernatant. On the assumption that some of the toxin was leached from cells into the culture media, the supernatant was examined as a toxin source. The supernatant from cultures in log phase was not toxic. Concentration of supernatant 20-fold in cellophane membranes by evaporation in a forced air draft was effective, as shown by bioassay. At this concentration, some toxicity was attributable to the extreme salt concentration, as shown by concentrated uninoculated media; however, the concentrated supernatant in which cells were grown was more toxic and caused more typical physiological symptoms than the concentrated pure medium. Attempts to separate the toxic principle from the saturated salt solution were unsuccessful.

Discussion

Our chief objective was to determine whether the toxin of California mussels is derived from *G. catenella* and is identical with the mussel toxin whose chemical and physical properties had been studied by other investigators.[6,7,8] The relationship between poisonous mussels and blooms of *G. catenella* in California waters is very good circumstantial evidence as to the origin of the poison, but it does not exclude the possibility that bacteria or other microorganisms normally accompanying *G. catenella* blooms are the source of the toxin.

Axenic *G. catenella* mass cultures often showed variation in production of toxin. The number of cells necessary to produce one MU varies from 7×10^4 to 2×10^6. This variation reflects our ignorance of the environmental factors for toxin production.

Purification of the toxin from *G. catenella* is difficult because of low toxin yield per cell, its high polarity, its water solubility, and its dialyzability. To obtain sufficient toxin it was necessary to harvest 12 Fernbach flasks every month. The toxin was extracted and stored frozen until sufficient quantities were accumulated. Chemical and physical properties of the mussel toxin were compared with this material.

G. catenella and mussel toxin are both dialyzable, have the same diffusion coefficients (according to E. J. Schantz, in a personal communication), *p*H stability, R_f values in paper chromatography, and color reactions with the Jaffe reagent. They also behave alike in ion-exchange adsorption. In similar salt solutions, or when mixed, both compounds exhibit the same patterns in electrochromatography. When injected intraperitoneally into mice, both induce the same physiological effects. These parallels indicate that the compounds are identical and that toxin found in California mussels has its origin in *G. catenella*. Schantz, in a study presented elsewhere in this monograph, reports that the poison found in the California mussel and the Alaskan butter clam are similar.

Acknowledgment

We are indebted to Edward J. Schantz, of the United States Army Chemical Corps, Biological Laboratories, Fort Detrick, Frederick, Md., for valuable assistance and for supplies of shellfish toxin.

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BIOCHEMICAL STUDIES ON PARALYTIC SHELLFISH POISONS

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The recent purification of the poisons from toxic California mussels, *Mytilus californiasus* Conrad and Alaska butter clams, *Saxidomus giganteus*, by combined efforts of workers at the University of California, Berkeley, Calif., Northwestern University, Evanston, Ill., and the Chemical Corps Biological Laboratories at Fort Detrick[1,2] has offered an opportunity to study the nature of these very toxic substances. These poisons were found to be among the most potent known to man, and it is evident from animal experiments that the purified poison from mussels is the principal cause of a food poisoning in humans commonly called "mussel poisoning," described by Meyer *et al.* in 1928.[3]

In cases where humans have had the misfortune to collect and eat toxic mussels, symptoms usually have occurred in one half to three hours. The symptoms begin by a numbness in the lips and finger tips, followed by progressive paralysis and death from respiratory failure in two to 12 hours, depending upon the magnitude of the dose. If a person survives for 24 hours the prognosis is good. Those who survive usually show no lasting effects from the ordeal. Meyer determined the lethal dose for humans by counting empty mussel shells, assaying the uneaten mussels left from fatal cases, and by interviewing survivors; he gave a minimum figure of about 20,000 mouse units (MU).[4] In terms of the purified poison this figure would amount to 3 to 4 mg. Canadian workers (Bond and Medcof[5] and Tennant *et al.*[6]) have found that the value may be much lower, and believe that lower doses may cause death or illness in persons who do not usually eat mussels or clams and have thus developed no tolerance to the poison. The intraperitoneal dose for the mouse is 0.18 μg., or 9 μg./kg. of body weight, and the intravenous dose for rabbits is 3 to 4 μg./kg.,[1] as Murtha reports elsewhere in this monograph. Doses for various animals and for man in terms of shellfish products are reviewed by McFarren *et al.*[7] The pharmacological action of the poison is described by Murtha in his paper in these pages.

A review of the developments leading up to the present knowledge of the chemical and physical nature of the poison includes work dating back to the year 1778, when da Costa* attributed the poisonous effect to the accumulation of copper salts in the shellfish. The first suggestion that the poison might be due to the presence of an organic compound was made by Christensen* in 1845, when he found that the poison was destroyed by ashing the shellfish. In 1862 Husemann* postulated that the toxicity arose from putrefaction in shellfish at certain times of the year, particularly during the warm summer months. In 1885 a mass intoxication from eating mussels, *Mytilus edulis*, occurred near Wilhelmshaven, Germany, and Brieger[8,9] claimed to have isolated from the toxic mussels a poisonous substance in the form of a gold salt, which he called "mytilotoxin." He gave no detailed data on the toxicity of the substance, but considered it a quaternary ammonium base and the pure posionous principle. Several attempts by later investigators, Thesen[10] and Muller,[11] to duplicate the work of Brieger failed to yield the gold salt. Salkowski[12] also attempted purification of the poison from the toxic mussels near Wilhelmshaven, but had little success. However, he did describe properties of the poison that enabled later investigators to make comparisons with the poison from other sources. In 1886 Wolff[13] found that the poison was localized in the "dark gland," or hepatopancreas.

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* Cited by Muller.[11]

One of the most puzzling phenomena associated with mussel poisoning was the unpredictable and sporadic occurrence of the poison. The only clue to its presence was when human cases of poisoning began to appear after eating mussels. Toxicity in the mussels usually appeared quite suddenly and, often within one to two weeks after a series of fatal human cases, mussels from the same location could be eaten again without harm. Progress on the investigation of the poison could be made only when an investigator was successful in collecting toxic material at the time of one of these outbreaks.

Although outbreaks of poisonings from eating mussels and clams along the Pacific coast have been recorded since 1793, certain serious outbreaks of mussel poisoning near San Francisco in the years 1927 onward gave Sommer and Meyer[14] and Sommer *et al.*[15] at the University of California an opportunity to investigate the source and nature of the toxic principle. These workers solved the puzzling mystery of the sporadic occurrence of the toxic mussels when they found that the poison in the mussels was associated with the occurrence of the dinoflagellate, *G. catenella*, and that these organisms were poisonous. Most shellfish consume plankton for food, and these workers found mussels to be toxic only when *G. catenella* were present in the sea water bathing the mussels; the toxicity of a mussel was proportional to the number of *G. catenella* cells found in the water and in the stomach of the mussels. Sommer[15] also cultured *G. catenella* in the laboratory and found that mussels placed in these cultures became toxic. When replaced in water free of *G. catenella* or in the cultures of nontoxic plankton, they lost their toxicity within a few days.

Through the work of Provasoli and his co-workers at the Haskins Laboratories, New York, N.Y., *G. catenella* has been obtained in pure culture free of bacteria, and it was found that the organisms still produced the poison (personal communication). Thus the poison must be a metabolic product of the organism, and not the result of a symbiotic effect of the bacteria normally growing with it.

The first thorough study of the chemical nature of the poison was begun under the leadership of Herman Sommer and his co-workers. Sommer and Meyer pointed out the similarity of the poison of California mussels and the Wilhelmshaven mussels, and showed that a basic organic compound could be extracted from the hepatopancreas of toxic mussels and from the toxic plankton. Muller,[11] working with Sommer, investigated various methods of purification and demonstrated the stability of the poison to oxidation in acid solution and its lability in alkaline solution. In 1944, Byron Riegel, D. Warren Stanger, and their associates at Northwestern University entered into a cooperative study with Sommer on the isolation and purification of the poison, and shortly afterward the Biological Laboratories of the Chemical Corps also took part in the cooperative study. Other groups later joined the study of the chemistry of the poisons. Among these were Herbert E. Carter and his associates at the University of Illinois, Urbana, Ill., and Oskar P. Wintersteiner and his associates at the Squibb Institute for Medical Research, New Brunswick, N.J.

The purpose of this paper is (1) to bring together in summary form for this monograph the published results of these cooperative studies, along with the results of other investigators, and (2) to present additional data important to our knowledge of the purification, purity, and chemical nature of these poisons. McFarren *et al.*[7] have reviewed the public health significance of these poisons, Courville *et al.*[16] have reviewed marine biotoxins in general, and Meyer[4] has reviewed mussel and clam poisons as a problem in food poisoning.

Collection of Toxic Shellfish

One of the most difficult problems in this study was to obtain sufficient toxic material to make a thorough investigation of the chemical and physical properties of the poison and the various procedures for isolation. Toxic shellfish have been found sporadically in many parts of the world, but certain areas, such as the Pacific coast of North America, the Bay of Fundy, the North Sea and English Channel, and regions of South Africa are troubled more or less chronically. It was felt that poison from different species of shellfish might be similar enough so that information obtained on the poison of one would be of value in the study of the poison of others. Three sources of poison therefore were obtained for this study: (1) California mussels; (2) Alaska butter clams; and (3) Bay of Fundy scallops.

The sporadic occurrence of toxic mussels made it necessary to carry on a continual assay program for toxicity in the mussel beds along the California coast. The search for toxicity was carried on from May through October by Sommer and interested volunteers at the George Williams Hooper Foundation, University of California Medical School, San Francisco, Calif. At the beginning of each minus tide period samples of mussels were collected and assayed. A microscopic examination of the water for the presence of *G. catenella* was also made at these times in an attempt to predict the occurrence of toxic mussels. To obtain toxic mussels it was necessary that the growth of toxic plankton coincide with the last four or five days of the minus tide periods so that the mussels could be collected. In most cases the toxic plankton culture would grow and recede within one to two weeks. When mussels were found to be sufficiently toxic (at least 4000 MU in a 100-gm. mussel), the digestive glands (hepatopancreas), which contained about 95 per cent of the poison, were removed and placed in acidified ethanol; when the purification process was started immediately the glands were placed in about 0.1 *M* HCl. About 200 million MU of poison were collected between 1944 and 1953, chiefly during the outbreaks in 1944 to 1948.

In contrast to mussels, the Alaska butter clams, as they become toxic, retain the poison in the siphons for long periods of time, which makes collections practical at convenient minus tide periods anytime throughout the year. Clams were collected, when the toxicity was 5000 or more MU per 100 gm. of siphons, from various areas of southeastern Alaska (Hood Bay, Chaik Bay, Robert Island, and areas near Ketchikan) with the help of the Fishery Products

Research Laboratory, United States Department of the Interior, and Alaska Experimental Commission, Ketchikan, Alaska. Clams in these areas are quite inaccessible and assistance from commercial canners was of great help, particularly in digging the many tons of clams required for this work. The siphons, which contain about 60 per cent of the poison in a clam, were removed, frozen, and stored until ground for extraction of the poison.

Toxic scallops, *Pecten grandis*, were obtained through the assistance of G. B. Reed and his associates, of the Canadian Defence Research Council, Ottawa, Ont., Canada, and were collected from the Bay of Fundy. The digestive glands that contained most of the poison were removed and stored in acid solution (pH 2 to 3) with 15 per cent ethanol to prevent fungal growth.

Methods of Assay

The first practical quantitative bioassay for the poison was developed by Sommer and Meyer.[14] They defined a mouse unit as the minimum amount of poison required to kill a 20-gm. mouse in 15 min. when 1 ml. of solution was injected intraperitoneally. Larger doses will kill a mouse in less time. The curve relating time of death to mouse units may be constructed from the following data. Death times of 4, 5, 6, 7, and 8 min. are equivalent to 2.5, 1.9, 1.6, 1.4, and 1.3 mouse units respectively. If the logarithm of the dose is plotted against the reciprocal of the time a straight line is obtained. The dose may be calculated directly from the equation

$$\log \text{dose} = (145/t) - 0.2$$

where t is the time of death in seconds and the death occurs between 240 and 480 sec. Solutions to be assayed should be between pH 3 and 4. The mouse units of poison per mg. of dry solids is referred to as the specific toxicity of a preparation. Medcof *et al.*[17] have described a similar assay for the poison in shellfish and Stephenson *et al.*[18] have studied the conditions for the assay in detail.

Assays on the purified poison were slightly different than on crude preparations, but consistent and accurate results were obtained when the dilutions were adjusted so that the time of death was between 4 and 8 min. and preferably between 5 and 7 min. These times represent the portion of the death-time response curve where the dose is most accurately determined from the time of death. The time of death is defined as the time from challenge to the last gasping breath of the mouse. The time to death is affected by the presence of salts and by ethanol. For example, 1 per cent of sodium chloride in the assay solution injected into the mice will increase the death time sufficiently to reduce the assay results by 50 per cent. Salt concentrations less than 0.1 per cent seem to cause no trouble. The presence of ethanol also increases the death time if it is present in concentrations greater than 3 to 5 per cent.

Studies in cooperation with Lewis, McFarren, Schafer, and Jensen, of the United States Public Health Service, Cincinnati, Ohio, have resulted in modifications of the assay procedure and include using the purified shellfish poison as a reference standard.[19,20] The mouse unit is a variable quantity depending upon species of mice, conditions of assay, and various other factors. By expressing the response of mice in terms of a definite weight of poison in the reference standard, the results of assays from various laboratories have been found to check very well. This procedure has been made the official method of assay by the Association of Official Agricultural Chemists.*

Purification of the Poisons

The basic nature of the poison in mussels led Sommer and his co-workers to investigate the use of cation exchangers for its purification. Muller,[11] working with Sommer, used Permutit in this manner and also precipitated the poison with rufianic acid and reinecke salt and obtained a preparation of mussel poison with a toxicity of about 500 MU/mg. Bendein and Sommer,[21] Monnier[22] and Sommer, Riegel and their co-workers[23-25] investigated various permutites (Decalso), as well as chromatography on carbon, and produced a preparation with a toxicity of about 1600 MU/mg.

Decalso was not an efficient cation exchanger for removal of the mussel poison from the crude extracts. If the amount of poison in the solids was less than 3 MU/mg., very little if any poison was removed by the Decalso and, consequently, it was of no value in removing clam poison from the extracts of the siphons. The crude extracts were made by grinding the dark glands of scallops and mussels or the siphons of clams, mixing with a filter-aid such as Celite 545, and extracting with water acidified so that the extract came through at about *p*H 3. Ethanol was added to the acidified water (15 per cent) to prevent fungal growth. Complete recovery of the poison was obtained in the extracts. Mussel extracts usually contained 4 to 8 MU/mg. of dry solids and extracts of clam siphons contained 0.5 to 2 MU/mg.

The carboxylic acid resins proved to be the ideal cation exchange resins for the purification of the clam and mussel poisons. Removal of the poisons from the crude extracts was 98 to 100 per cent, even from low potency material such as the extracts of clam siphons. The poison in a crude extract was absorbed on the sodium form of Amberlite IRC-50. The resin was then washed with water, followed by washing with an acetic acid-sodium acetate buffer at *p*H 4.0, which removed over 99 per cent of the inert solids with less than 5 per cent of the poison. The poison was then fractionally eluted from the column with 0.5 *M* acetic acid. The best fraction contained over 90 per cent of the poison with a specific toxicity of 400 to 500 MU/mg. Chromatography of these fractions on the acid form of Amberlite XE-64 resulted in preparations with a potency of about 3000 MU/mg. Further chromatography of the best fractions from the XE-64 column on acid-washed alumina in absolute ethanol brought the toxicity to 5500 MU/mg. Repeated chromatography of different types failed to increase the specific toxicity.[2] Over-all yields of poison of about 50 per cent were obtained, and were increased to 70 per cent when the side fractions from the chromatographic columns were reworked. This procedure[1] proved most satisfactory for handling the large amounts of starting material needed to obtain a gram of poison. In some cases it was necessary to process up to 600 pounds of clam siphons (8 tons of clams) to obtain one gram of poison (assuming 50 per cent over-all yield of the poison in purified form).

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* Samples of the purified shellfish poison for standardization of animal assays and similar work are available from the Milk and Food Division, Sanitary Engineering Center, United States Public Health Service, 7626 Columbia Parkway, Cincinnati, Ohio.

Difficulty was encountered in the use of these resins for the purification of the poison from scallops. This poison was adsorbed onto the sodium form of Amberlite IRC-50, but most of it was eluted above *p*H 4 with the bulk of the impurities, whereas 95 per cent of the clam and mussel poisons were eluted below *p*H 4. The difference in behavior may be interpreted to mean that the chemical properties of scallop poison are different from the others or that the impurities in the scallop extracts affected the character of the resin in such a manner that the poison was not bonded firmly to the resin. The possibility that the scallop poison is actually different chemically is supported by experiments in which equal numbers of units of purified clam poison were added to crude preparations of scallop poison and processed on the sodium form of Amberlite IRC-50, using acetic acid at *p*H 4.5 and *p*H 3. The total units were about equally eluted at the two *p*H values. It was not possible to determine which poison came off the resin at a particular *p*H, but in a mixture whose impurities were common to both poisons it appears reasonable that all of the poison should have been eluted at *p*H 4.5, if impurities were the determining factor. These experiments do not exclude the fact that the impurities are causing the trouble, but do give support to the possibility that the pK_a values of scallop poison may be different than those of clam or mussel poisons. Because of the difficulties encountered with scallop poison on the resin the purification was not pursued further.

Purification of clam and mussel poisons was achieved by two other procedures that may be of value to those interested in this field of work. The first of these, developed by Mold[26] and Wikholm[27] working with Riegel, was by successive crystallization of partially purified mussel poison (1000 to 2000 MU/mg.) as the helianthiate, followed by chromatography of the hydrochloride salt of the poison on Norit A.[24,2] The product obtained had a specific toxicity of 5000 MU and a specific rotation of 122° to 130°. Mold and Bowden, working with me,[1,2] also studied purification by countercurrent methods. These procedures were complicated by the fact that the hydrochloride salt of the poison is soluble only in water, methanol, and, to some extent, in ethanol, and is quite insoluble in all solvents immiscible in water. However, by working at *p*H 8 and at a cold temperature (5° to 10° C.), satisfactory distribution was attained in a system consisting of *n*-butanol, ethanol, 0.1 *M* aqueous potassium carbonate, and α-ethylcaproic acid in a volume ratio of 146:49:200:5, with the aqueous layer adjusted to *p*H 8. Crude preparations could not be purified, but a partially-purified preparation of either clam or mussel poison at about 3000 MU/mg. could be increased to a potency of about 5000 MU/mg. in 24 transfers, with a yield of about 10 per cent. Chromatography on acid-washed Norit A was then necessary to remove the salts. This product had a specific optical rotation of 130°.

The greatest problem in the purification of the shellfish poisons appears to be the separation of a series of nontoxic basic substances closely related to the poisons.[28] Complete separation of a single component under these conditions is very difficult, but it is believed that the clam and mussel poisons purified to a specific toxicity of 5500 MU and a specific rotation of 130° represent a practical limit in this type of purification. Dried preparations of the poison often appeared crystalline, but no crystallization from solutions as simple salts was obtained.*

Studies on the Purity and Authenticity of Clam and Mussel Poisons

The best evidence for the purity of the poisons was obtained by countercurrent distribution studies.[2] Using the solvent system found best in one of the purification methods, Mold, Bowden, and their co-workers[2] demonstrated that the purified poisons separated into two components designated as *A* and *B*. When either component was isolated and run through the distribution again, a redistribution occurred in exactly the same manner as the original. The distributions were followed by toxicity and total nitrogen. These data suggest that the poisons exist in two tautomeric forms. One form, *A*, was found to exist in greater quantity and had a specific toxicity somewhat greater than 5500 MU/mg., while the other form, *B*, had a specific toxicity somewhat less than 5500 MU/mg. However, when either form was allowed to stand for a few hours in acid solution, the toxicities came to the average value of 5500 MU/mg. It was found that when the poisons were reduced to the dihydro derivatives and studied, the distribution showed only one component and fit a theoretical distribution for a single substance. The distribution of the dihydro derivative of the poisons was followed by nitrogen analyses only because the derivatives are nontoxic. The properties of the dihydro derivatives of the poisons will be described subsequently. Apparently the reduction stabilized the compound in one tautomeric form, which distributed as a single component. It must be assumed, therefore, that the poison in its tautomeric form also is a single substance.

The fact that several methods of purification yielded products with a specific toxicity of about 5000 MU/mg. and specific optical rotation of about 130° gives supporting evidence that they are pure. The degree of purity that can be stated on the basis of the distribution studies depends, of course, on the accuracy of the analytical procedures. In this case an allowance of ±5 per cent must be made for experimental error so that the purity should be between 95 and 100 per cent.

The possibility of the purified poisons being changed through the isolation procedure has been studied. Japanese workers[29a-29b] have raised the same question in regard to the purified poisons isolated by the above procedure. Reasonable proof, however, can be given that the poisons are not altered during the isolation procedure. First, the general symptoms of mice and rabbits when challenged with crude and purified preparations of either clam or mussel poisons are essentially the same. Mice showed a slightly inconsistent response to the highly purified poisons when administered in the most dilute lethal dose, but this inconsistency was eliminated if some of the nontoxic impurities from the isolation procedure were added to the solution being assayed. Second, the total recovery of units of poison in all fractions checked, within experimental error, with the total units indicated by the assay on the pooled digestive glands of mussels or siphons of clams. This recovery was the same whether glands or siphons were extracted with water at *p*H 2 to 3 or with aqueous acidified ethanol. It seems reasonable that any change in the molecule affecting the toxic structure would be reflected in the animal response and total recovery of units. Third, the diffusion coefficients of the crude and purified poison preparations were essentially the same (4.9×10^{-6} sq. cm./sec.), as measured by mouse assay with the Northrop diffusion cell and by diffusion into agar blocks. The diffusion into agar was carried out so that the agar could be sliced, each slice assayed for the poison, and the form of the diffusion curve determined. Perfectly normal curves were obtained for the diffusion of purified poison in water (*p*H 3) and poison in crude water extracts (*p*H 3), indicating that the crude preparations, within the limits of diffusion measurements, contain only one poisonous substance.

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* R. A. B. Bannard and his co-workers in the Department of National Defense, Ottawa, Canada, have reported recently by personal communication the development of a chromatographic technique on paper that yields a purified preparation of poison from clams, similar in potency and properties to that described in this paper.

Biochemical Properties of Purified Clam and Mussel Poisons

A study of the biological, chemical, and physical properties of the purified clam and mussel poisons indicated that the two poisons are very similar, if not identical. Details of the chemical and physical properties have been reported.[2,30] In summary both are basic substances forming salts with mineral acids. The dihydrochloride salts are white hygroscopic solids, very soluble in water, soluble to some extent in methanol and ethanol, and insoluble in all lipid solvents. They have a specific optical rotation of 130°, show no absorption in the ultraviolet, and have the same infrared spectra with strong absorption at 3, 6 and 9 μ. Two base functions are present in equivalent amounts, pK_a 8.1 and about 11.5. The molecular formula was found to be $C_{10}H_{17}N_7O_4 \cdot 2HCl$, with a molecular weight of 372.

As a strongly basic substance one would suspect that the poison might be classed as an amine or a quaternary ammonium compound. However, studies on the methylation of the poisons indicated that such structures are not present. The poisons react with certain aromatic nitro compounds to form colored complexes in much the same way as creatinine reacts with dinitrophenol in Jaffé's test, and with dinitrobenzoic acid in the Benedict-Behre test. In fact, the close similarity of the poisons to creatinine on a molar basis in the Jaffé and Benedict-Behre tests suggests that their structures, in part, must be very similar. Other compounds, such as hydantoin and certain barbituric acid derivatives, that give positive test reactions showed marked differences from the poisons and creatinine in absorption characteristics of the color complexes. The Weber nitroprusside test, commonly used for guanidine, is also positive for the poisons, but the Irrevere-Sullivan test for creatininelike compounds was found to be negative. The Sakaguchi test is negative, indicating that a free guanidinium group such as exists in arginine is not present in the poison. Negative tests were also obtained for the Weygland and Csendes test for enols of 1, 3, diketones, the Fearon and Mitchell test for primary and secondary alcohols, and the Benedict test for reducing sugars.

Howard and Mold[30] at Fort Detrick found that the purified poisons could be reduced with hydrogen at a pressure of one atmosphere in the presence of platinum black to produce a dihydro derivative. This derivative was found to be nontoxic. One mole of hydrogen was taken up for each mole of poison. The extent of hydrogenation correlated directly with the loss of toxicity in all cases. Benedict-Behre and Jaffé tests were found to be negative for the dihydro derivative and correlated directly with the toxicity throughout the reduction. The Weber test, however, remained positive during this treatment. Wintersteiner, Dutcher, and Walters,[30] at the Squibb Institute, found that when the poisons were exposed to oxygen in alkaline solution, the toxicity decreased in direct proportion to the oxygen uptake and, in this case, the Benedict-Behre and Jaffé tests also correlated directly with the bioassay for toxicity. The Weber test remained positive. After the poisons were reduced they would not take up oxygen in alkaline solution. The results indicate that the toxicity of the poison is dependent upon the presence of an unsaturated bond that is reduced with hydrogen or oxidized with oxygen in alkaline solution. The good correlation of the Benedict-Behre and Jaffé tests under these conditions has suggested their possible use as a quantitative chemical test for the poisons.[30] Such a test would be of practical importance, particularly where mice are hard to get and keep in condition for assay work. McFarren, Campbell, and Lewis of the Sanitary Engineering Center, in cooperation with myself, have studied the application of the Jaffé test to the determination of the poison in fresh clams and in clam products.[31] These products were found to contain substances other than the poison that react in the Jaffé test, but fortunately the color complexes formed in the test by the interfering substances differ in their characteristics sufficiently to allow the poison to be determined quantitatively in their presence by measuring the absorption of the colors at two different wave lengths. The procedure now appears practical, at least under certain laboratory conditions, for the chemical determination of the poison in fresh and processed clams.

Successful attempts to produce derivatives or degrade the poison molecule to derivatives suitable for structural studies have not been reported at the time of this writing. All derivatives have been too large or too small for this purpose. Recent studies by H. R. Rapoport, University of California, have indicated some success (personal communication). The dihydro derivative ($C_{10}H_{19}N_7O_4 \cdot 2HCl$, mol. wt. 374), because of its complete loss of toxicity resulting from a small chemical change, has been studied extensively in my laboratory. It has a specific optical rotation of 130°, no absorption in the ultraviolet, and pK_a values of 8.3 and about 11.5. In these properties it is similar to the poison. Only slight changes in the infrared spectra were observed upon reduction, indicating a probable change in an amidic group.[30] The striking similarity of the poison to creatinine in its reactions with certain aromatic nitro compounds, mentioned previously in this report, suggests that the tautomerism responsible for the reactions in the two compounds should be similar. However, creatinine could not be reduced to any measurable extent under the same conditions in a Warburg manometric flask that allowed complete reductiod of the unsaturated bond involved in the tautomerism in the clam or mussel poisons. Oxidation of the poison with periodate yielded guanidopropionic acid, ammonia, and carbon dioxide.[32,33] Oxidation with potassium permanganate produced guanidine and urea, and strong acid hydrolysis produced guanidine in addition to ammonia and carbon dioxide.[32,33] Oxidation with air in alkaline solution at room temperature yielded a variety of substances. None of these substances has been isolated and characterized, but the ultraviolet absorption and fluorescent properties of certain fractions of the mixture indicated the presence of pteridinelike compounds.[32,33] All of the nitrogen in the poisons is reduced to ammonia in the Kjeldahl procedure, and about one half of the nitrogen can be liberated in the form of ammonia by heating the poisons directly in alkaline solution. No attempt is made here to give details on the various properties of the poisons or to interpret these findings in terms of possible chemical structures. The detailed studies of the groups at the University of California, University of Illinois, Northwestern University, Squibb Institute for Medical Research, and at Fort Detrick have been compiled by Mold, Howard, Bowden and myself in Chemical Corps reports.[32,33]

Neither of the purified poisons was found to be detoxified in the presence of saliva, pepsin, or trypsin. No detoxification was observed in solution in whole blood or plasma, or in urine. Ground mouse liver, skeletal muscle, or kidney, when mixed with an equal volume of water containing purified poison to form a brei containing 100 MU/ml., brought about less than a 5 per cent removal of the poison from the fluid bathing the tissues. The results were the same with sliced tissues. Ground hepatopancreas from nontoxic mussels produced similar results, but the hepatopancreas from a medium-sized toxic mussel that had fed on *G. catenella* may yield several thousand mouse units of poison per gram of wet tissue (about 1 ml./gm.) when extracted with slightly acid solutions. Apparently there is no substance within the digestive gland of the mussel that combines with and immobilizes the purified poison under the conditions of these experiments. The process by which the mussels or clams remove the poison from poisonous plankton, bind it in the hepatopancreas or siphon, and eventually dispose of it is an interesting study. These processes, however, may be dependent upon the conditions within the intact hepatopancreas.

The action of the poison has been described as similar to that of curare, but the substances that inhibit the action of curare do not inhibit the action of the poison, as reported by Murtha elsewhere in this monograph. No antidote for the poison is known. H. A. Neufeld and I, in an unpublished study, have investigated to some extent the action of the poison on the mechanisms of muscular contraction. The contraction of isolated muscle fibers (psoas from rabbit) in the presence of ATP and magnesium ions, as described by Szent-Gyorgi,[34] was not inhibited by concentrations of mussel or clam poisons up to 1 mg./ml. When the poison was introduced into a Warburg flask containing respiring diaphragms, freshly removed from mice, no change in the rate of oxygen consumption was noted. J. O'Neill and his associates have found that the mechanism converting ADP to ATP from creatinine phosphate is not inhibited by the poison (personal communication). The administration of atropine to mice or rabbits challenged with shellfish poison did not change the symptoms or death times, and this fact would indicate that the poison is not a cholinesterase inhibitor. Bolton *et al.*[35] have published similar observations from studies on end-plate potentials.

At this time I know of no reports of a specific biochemical reaction involved in nerve or muscle physiology that might explain the specific action of the poisons. The poisons, therefore, should be valuable tools for further studies in these fields (Hutner and McLaughlin).[36]

It was stated previously that the primary source of mussel poison was in the dinoflagellate *G. catenella*, upon which the mussels feed. The actual primary source of the poison in the Alaska butter clam is not known. L. Foster and Sommer have examined the water in areas of southeastern Alaska where clam beds have become toxic, and claimed to have found only an occasional *G. catenella* cell (personal communication). These waters support the growth of a great number of plankton, but none of those examined by these workers was found to be toxic. While collecting toxic clams in Alaska I collected mussels from rocks near clam beds, but very seldom found any poison in them. This fact would indicate that they were not consuming any appreciable number of toxic plankton in their food supply. One explanation for the toxic clams existing with nontoxic mussels might be that the low intake of toxic plankton over a period of many months builds up appreciable amounts of poison in the siphons, whereas the mussels would excrete or destroy this small amount from day to day. However, the possibility that the poison may have another source should not be overlooked.

As far as I know, toxins or poisons produced by the different species differ somewhat in at least some of their properties and in their chemical structure. Studies presented elsewhere in these pages by workers at the Haskins Laboratories (Burke *et al.*), point out the chemical similarity of the poison in *G. catenella* to the poison isolated from mussels. The close similarity of the chemical and physical properties of the clam and mussel poisons would, therefore, suggest that clam poison might also have its origin in *G. catenella*. In this respect it would be of interest to study further the poison from scallops that become poisonous from the consumption of *Gonyaulax tamerensis*.[37] Until more is known about the organisms in the water, the currents and tides carrying these organisms, and the environment in which the clams live, no conclusions on the origin of the poison in these clams can be drawn. This problem is of considerable importance to the economy of Alaska.

Much work still needs to be done on the chemical structure of these poisons and on their mechanism of action before any clear understanding of their nature can be elucidated. It is hoped that the availability of the purified poisons and the information presented and referenced here will help others pursue studies on these very interesting and unusual substances.

Summary

The past work on paralytic shellfish poisons is reviewed briefly. The poisons from California mussels and Alaska butter clams have been obtained in pure form. Purification was achieved by chromatography of crude extracts on carboxylic acid resins (Amberlites IRC-50 and XE-64) followed by chromatography on acid-washed alumina. Attempts to purify scallop poison by this procedure were not successful, and the indications are that some of the chemical properties of scallop poison are different from those of clam and

mussel poisons. The original source of these poisons is from toxic plankton that the shellfish consume as food. Clam and mussel poisons have been found to be similar in their chemical, physical, and biological properties. The poisons are basic nitrogenous substances soluble in water and lower alcohols and insoluble in all lipid solvents. They are stable in acid solutions but labile in alkaline solutions. The molecular formula is $C_{10}:H_{17}N_7O_4 \cdot 2HCl$. The molecule is readily reduced with hydrogen to form a nontoxic dihydro derivative. Various oxidizing and hydrolytic agents yield guanidopropionic acid, guanidine, urea, ammonia, and carbon dioxide.

The poisons are among the most potent known to man. Their action appears to be at the myoneural junctions, but no biochemical mechanisms studied thus far in muscles or nerves explain the specific action. The chemical structure and specific mechanism of action are problems remaining to be solved.

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MASS CULTIVATION OF SOME PHYTOPLANKTONS*

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INTRODUCTION

Food for the great productive fisheries and, indeed for marine life generally, centers around the utilization of solar energy by photosynthetic organisms, which are the grass of the oceans. Marine productivity to a great extent is a function of the number, type, and photosynthetic efficiency of such solar machines. Some of the variables controlling the growth of oceanic phytoplankton are inorganic nutrients, sunlight, water clarity, and organic growth factors. Many investigators have raised the question of the production by phytoplankton of exocrines.[1-5] The question has been put in many ways, but essentially it reverts to Putter's hypothesis,[6] which deals with the production of metabolically active products produced *in vivo* in microquantities, yet affecting cell, tissue, organ, and organ system. It is suggested that these exocrines may act as micronutrients, metal chelators, cellular toxins, mitotic inhibitors, hormones, or in other ways.

Recent work with axenic cultures of a wide variety of marine diatoms, cryptomonads, chrysomonads, euglenoids, and dinoflagellates demonstrates absolute *in vitro* requirements for vitamin B_{12}, thiamine, and biotin,[7-10] paralleling similar requirements in fresh-water organisms.[11,12] A need for growth factors implies production. Recently Ericson and Lewis,[13] Burkholder and Burkholder,[14] and others[15-19] have isolated organisms producing appreciable B_{12}, and have identified in raw sea water a variety of compounds, including citric, malic, and ascorbic acids, rhamnosides, B_{12}, and carbohydrates. Vallentyne[20] has compiled the record of organic matter in lakes and oceans. Fogg and Westlake[21] show extensive production of peptides by algae, which they believe to be important metal chelators, serving as "reservoir-metal-complexes." As for the production of carbohydrates, Allen[22] has demonstrated that carbohydrate secretions account for 10 to 45 per cent of the total soluble organic production of a *Chlamydomonas*. Guillard and Wangersky[23] investigated the carbohydrate production of 16 organisms and noted that the chrysomonad *Prymnesium parvum* secretes ~120 mg./l., and a dinoflagellate ~5 mg./l. Lewin,[24] in an analysis of 18 organisms comprising unicellular and colonial forms, identified fucose, mannose,

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rhamnose, and glucose as components of exogenously-produced polysaccharides. Fogg and Boalch[25] demonstrated that the brown alga *Ectocarpus* produces nitrogenous extracellular material much like that reported by Fogg and Westlake.[21] Armstrong and Boalch[26] report production of volatile organic matter from algal cultures and sea water. The production of a low-weight organic base by the dinoflagellate *Amphidinium carteri* is also recorded.[27]

The present investigation is an outgrowth of our interest in productivity, particularly in tropical waters, and the elaboration of toxic materials by members of various algal groups. In this paper we enter a phase of ecology represented by the interchange of metabolites among members of the phytoplanktonic community. We shall deal with the pouring out of astonishing amounts of carbohydrate by a dinoflagellate isolated from tropical waters. As will be shown, the amount given off immediately drew our attention to the possibility of quantitative analysis of the substance. As production of dissolved organic matter of algal origin represents energy fixed by photosynthesis, it was interesting to determine the amount, kind, and conditions under which such substance(s) were produced. Furthermore, the inherent difficulties in extraction of material from large volumes of raw sea water, in which exocrines are diluted manifold, are overcome by the techniques of axenic culture.

METHODS AND MATERIALS

The origin of the cultures used was:

Dinoflagellates. Katodinium dorsalisulcum, from waters off Bimini, British West Indies;[28] *Gyrodinium* sp., from bloom off Copiague, Great South Bay, Long Island, N. Y.; and *Gymnodinium* sp., from water samples, Bahia Fosforoscente, Puerto Rico.

Chrysomonads. Monochrysis lutheri and *Prymnesium parvum* maintained at the Haskins Laboratories;[9] *Monochrysis*, from a tide pool in England; and *Prymnesium* from a coastal fish farm in Israel.

Diatom. Melosira sp., from a Long Island Sound bloom.

Culture Methods

The *Gyrodinium*, *Gymnodinium*, and *Melosira* were obtained in axenic culture by methods previously developed.[9,29] Stock cultures of all organisms were routinely maintained in an enriched marine medium designated as MKD (TABLE 1). The medium was dispensed as 10-ml. amounts in 20 × 25 mm. borosilicate test tubes. Transfers were made approximately every 30 days. For mass cultures, air, sometimes enriched with 4 per cent CO_2, was bubbled under pressures of 5 to 35 psi. For large-scale production of carbohydrate, conical 2.5 l. flasks or 5 l. bottles were used. *K. dorsalisulcum* polysaccharide production flasks contained 1.5 l., and the bottles contained 4.2 l., of medium MMK (TABLE 1). For examination of crude carbohydrate material, medium MDV (TABLE 1) was developed to minimize the effect of substances interfering with the color test. All containers were cotton plugged and illuminated by banks of 40 w. cool white reflector-type fluorescent lamps of 800 to 1200 ft-c. Except in one instance, all cultures were grown at 22 to 28° C., maintained by room air conditioning. Occasionally lights were turned off for short periods (1 to 8 hours) when the cooling capacity of the air conditioner was taxed.

Nutritional Studies

Nutritional studies of *K. dorsalisulcum* were carried out in test tubes prepared as before,[9] with the additional precaution of washing the tubes and their plastic caps in 0.1 per cent NaOH and rinsing in distilled water until the *p*H of the rinse water was neutral. Cell counts were made with a standardized hemocytometer technique.

TABLE 1
MEDIA FOR MARINE ALGAE

	MKD*	MMK†	MDV‡
NaCl	2.4 gm.	2.5 gm.	1.8 gm.
$MgSO_4 \cdot 7H_2O$	0.9 gm.	0.9 gm.	0.5 gm.
KCl	0.07 gm.	0.07 gm.	0.06 gm.
Ca (as Cl^-)	30.0 mg.	30.0 mg.	10.0 mg.
$NaNO_3$	5.0 mg.	—	—
NH_4Cl	—	—	0.5 mg.
$Na_2SiO_3 \cdot 9H_2O$	1.0 mg.	1.0 mg.	20.0 mg.
P-II metal mix§	0.3 ml.	3.0 ml.	3.0 ml.
Nitrilotriacetic acid	20.0 mg.	20.0 mg.	0.3 µg.
B_{12}	1.0 µg.	—	0.1 µg.
Vitamins No. 8§	—	—	0.1 ml.
Biotin	1.0 µg.	0.1 µg.	—
Thiamine HCl	0.01 mg.	0.01 mg.	—
Pyridoxine HCl	2.0 µg.	1.0 µg.	—
Folic acid	2.0 µg.	—	—
K_2HPO_4	1.0 mg.	1.0 mg.	3.0 mg.
Urea	0.1 mg.	—	—
DL-alanine	5.0 mg.	50.0 mg.	1.0 mg.
Na_2 fumarate	5.0 mg.	5.0 mg.	—
$(NH_4)_2SO_4$	0.1 mg.	—	—
D-ribose	5.0 mg.	5.0 mg.	—
Sorbitol	—	5.0 mg.	—
Tris§	0.1 gm.	0.1 gm.	10.0 mg.
H_2O	100 ml.	100 ml.	100 ml.
*p*H	7.6–7.8	7.4	7.8–7.9

* Maintenance medium for extended logarithmic growth.
† Mass culture medium for maximum polysaccharide production.
‡ Medium designed specifically for *N*-ethyl carbazole test.
§ For details see McLaughlin and Zahl.[29]

Antibiotic Effect

Antibiosis by mucous material from *K. dorsalisulcum* was tested by the Vincent and Vincent disc method.[30] The test organisms were a heterogeneous group of 36 littoral and benthic marine bacteria.

Separation of Cells

All media were obtained cell-free by centrifuging the supernatant at 15,000 rpm, in a Serval continuous-flow (20 ml./min.) centrifuge. To free mucoid-embedded cells from *K. dorsalisulcum*, air under pressure of 30 psi was bubbled through the mucoid mass before centrifugation.

Treatment of K. dorsalisulcum Supernatant

The addition of 300 ml. of 95 per cent ethanol per 100 ml. of supernatant threw down a stringy mucilaginous precipitate indicative of a polysaccharide with linear structure.[31] The precipitate was separated by filtration through Whatman No. 40 paper, washed 6 times in ethanol, and stored over Drierite in a vacuum desiccator. Samples were worked up by hydrolysing 50-mg. portions with 2.0 ml. of 0.1 per cent sulfuric acid in sealed ampules for various times, essentially in the manner described by Lewin.[24] The hydrolysates were concentrated to dryness under vacuum, taken up in pyridine, spotted on sheets of Whatman No. 4 paper, and run as descending chromatograms in various solvent systems (TABLE 2). The chromatograms were developed by dipping through aniline diphenylamine in acetone, acidified with phosphoric acid, and heated until color spots appeared.

TABLE 2
PAPER CHROMATOGRAPHY OF ETHANOLIC PRECIPITATE
FROM *K. DORSALISULCUM* SUPERNATANT

Hydrolysis time (hours)	Solvent system	R_g* values for hydrolysis products:			R_g values of unknown
		Glucose	Galactose	Fructose	
2	IPrAq†	—	—	—	15, 200
	PhAq‡	—	—	—	23
4	IPrAq	—	85	104	55, 200
	PhAq	—	113	140	—
6	IPrAq	99	83	105	200
	PhAq	100	115	143	—
12	IPrAq	98	85	103	200
	PhAq	101	115	145	—

* $R_g = \frac{\text{distance substance travels from the origin}}{\text{distance glucose travels from the origin}} \times 100$. Controls agreed with hydrolysis products.

† IPrAq = isopropanol, water (160:40 v/v).

‡ PhAq = phenol, water (160:40 w/v).

Test for "Carbohydratelike" Activity

All mass cultures were tested with *N*-ethyl carbazole.[19] The supernatant cleared by centrifugation was further clarified by passage through an HA millipore filter (0.45 μ), which had been pretreated by washing with double-distilled water to remove interfering substances.[23] The filtered supernatant was stored at −4° C. The *N*-ethyl carbazole reagent, twice recrystallized from ethanol-water, was prepared before each test run. Absorption measurements were made at 562 mμ, using a Beckman model DU spectrophotometer adapted to take a 100-mm. cell. All glass used was "cured" by soaking in 25 per cent sulfuric acid for 24 hours, and the reactions were carried out in "low actinic" (red) 24-ml. test tubes. The reference standard was glucose in algal medium MDV (TABLE 1). Standard concentration curves were run for each supernatant tested. To avoid oxidation of samples, 1.0 ml. of mineral oil was added to the reaction vessel, providing a thin oil film over the liquid surface.

Demineralizing of K. dorsalisulcum Polysaccharide

Cellophane tubing* was used to remove trapped or chelated metals from the mucoid mass. The tubing containing crude polysaccharide was dialyzed against running tap water. Weight loss was used to estimate the noncarbohydrate fraction; no attempt was made to determine the metals lost through dialysis.

RESULTS

Nutritional Studies on K. dorsalisulcum

In attempts to improve growth, carbon, nitrogen, and phosphate sources were examined. Nitrate and ammonium nitrogen were utilized. Amino acids appeared favorable for polysaccharide production. Alanine, glycine, glutamic acid, methionine, and proline, individually and collectively, not only stimulated growth, but gave an average of 20 to 30 per cent more polysaccharide. *Katodinium* grew well in the presence of CO_2; glucose, maltose, sucrose, acetate, fumarate, succinate, sorbitol, and alanine in various combinations improved the growth and longevity of cultures. Such organically enriched cultures remained viable for 4 to 6 months, while others not so enriched died in 2 to 3 months. Because of the difficulty of separating organisms for inocula from the mucoid mass, carry-over obscured the vitamin requirements. B_{12} alone and in combination with thiamine and biotin gave greater total growth over long periods. The optimal NaCl concentration was 2.5 per cent. The salt tolerance ranged from 1.8 to 4.0 per cent (NaCl) when cells were directly inoculated from conversation medium MKD (TABLE 1). The optimal *p*H range with the media used was 7.4 to 8.2. Organic phosphate sources were no better than inorganic, but aided solubilization of metals.

Mass Culture

With an initial inoculum of 3 to 15 cells/ml. of culture, final cell counts ranged from 4 to 28 million per liter, depending on variables such as evaporation, temperature, and aeration. Cells inoculated directly into media with NaCl concentrations of >4.5 per cent lysed, but if through slow evaporation the salt concentration reached 7 to 8 per cent, cells remained viable, the mucus seemingly acting protectively. TABLE 3 illustrates some typical tests of variables possibly affecting carbohydrate production. Greatest production followed large inocula and constant aeration at 1500 to 2000 ft-c (the highest illumination attempted). CO_2-enriched air did not appreciably help carbohydrate production in the media tested. Apparently, after growth to an optimal cell concentration, the mucoid material inhibits further division, and the cells make mucus instead of putting energy into reproduction. When the cells have been grown at room temperatures and good growth densities are reached, removal to lower temperatures (17° C.) permits cell division but inhibits mucus production (TABLE 3). We have been unable to grow organisms at initial temperatures below 22° C. Once cultures have reached densities of 3000 to 7000/ml. (with accompanying mucoid production) they remain viable at temperatures as low as 10° C., the mucoid material again acting as a protective coating. In stationary cultures, after 10 to 20 days, the oxygen produced in photosynthesis becomes entrapped in the dense mucoid mass. As the cell growth and mucoid mass initiate on the bottom of the vessel, the trapped oxygen causes the gelatinous mass to float. As more oxygen is produced the entire mucoid mass starts a slow ascent from bottom to top of the vessel. During ascent the area is filled in from below with polysaccharide, and the end result is gelation of the entire medium.

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* No. 8-667, Fischer Scientific Co., New York, N. Y.

TABLE 3

PRODUCTION OF POLYSACCHARIDE BY MASS CULTURES OF *K. DORSALISULCUM* UNDER VARYING PHYSICAL CONDITIONS IN MEDIUM MMK

Cell count/ml.		No. days continuous incubation*		Dry wt. (gm.) polysaccharide/l.
Initial	Final	Aerated	Stationary	
3 to 15	5×10^3	15 (24° C.)	15 (24° C.)	1.4
3 to 15†	24×10^4	30 (24° C.)	—	2.6
3 to 15	2×10^3	—	30 (24° C.)	0.6
3 to 15	9×10^3	15 (24° C.)	15 (17° C.)	1.4

* Cultures were always aerated first, then allowed stationary growth.

† This culture lost two thirds of its volume in evaporation, and required 20 to 30 psi pressure to force air through the heavy, syrupy medium.

Cells trapped in the mass are nonmotile (FIGURE 1), but if the mass is violently disturbed (by aeration or otherwise) they quickly exhibit typical dinoflagellate motion.

Antibiotic Effects of Katodinium Polysaccharide

The results of testing the crude mucoid material against 36 marine bacteria are as follows: zone of partial inhibition, 9 cultures; stimulation, 18 cultures; change in colony size and/or morphology, 4 cultures; and no effect, 5 cultures.

ANALYSIS OF *K. DORSALISULCUM* POLYSACCHARIDE

Preliminary Tests

Tests on the raw material and the supernatant for reducing sugars were negative. An elemental combustion analysis yielded these values for the dried ethanol-precipitated material: C, 5.45 per cent, H, 2.06 per cent, O, 18.10 per cent, S, 6.11 per cent, N, 0.00 per cent, and ash, 73.27 per cent. Dialysis of the precipitated material in cellophane membranes against distilled or tap water for 24 hours showed a weight loss of ~72 per cent.

Chromatograms of Polysaccharide

On the basis of ash determination, dialysis, and subsequent chromatograms, 25 per cent of the mucoid material was estimated to be carbohydrate. Chromatograms containing samples that were acid-hydrolysed for 2, 4, 6, and 12 hours showed glucose, galactose, and fructose present. Further hydrolysis did not yield additional detectable products. Partial hydrolysis for 4 hours indicated fructose, galactose, and a spot corresponding to maltotriose. Samples hydrolysed for 2 hours were negative for mono-, di-, or trisaccharides (TABLE 2).

All chromatographic runs showed an unidentified yellow spot that moved with the solvent. This spot could be removed by treating the hydrolysate with activated charcoal before applying the sample. This spot was eluted from the paper with ethyl acetate, reconcentrated, and reworked on Whatman No. 3 paper in a solvent system of water, tertiary amyl alcohol, pyridine, and diphenylamine (25:25:25:1). Examination of strips under UV (3600 Å) showed fluorescent bands similar to those described by Shapiro[32] for organic acids. Ethyl-acetate extracts of raw supernatant had similar bands.

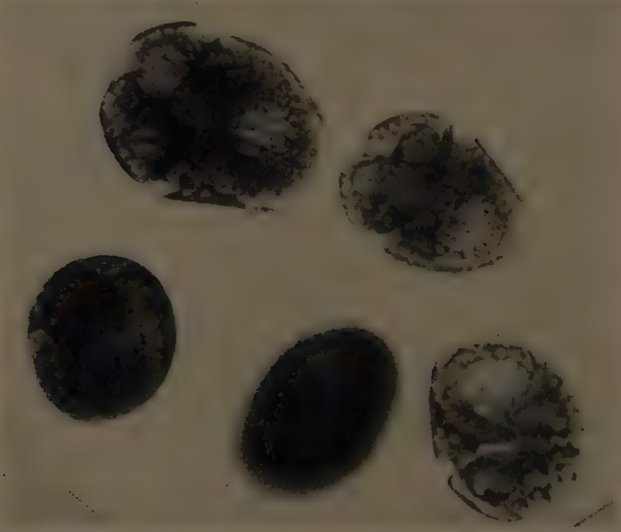

FIGURE 1. *Katodinium dorsalisulcum* cells entrapped in polysaccharide material. Original magnification ×400.

N-Ethyl Carbazole Test of Cell-Free Supernatants

As reported by others[19,23,32] this reaction is nonspecific and may give positive reading with many reagents. These authors have interpreted the color intensity in terms of mg. of some particular carbohydrate from a standard sugar curve. Lewis and Rakestraw[19] and Collier[33] have reported the reaction to be nonspecific, difficult to reproduce, and affected by many organic and inorganic reagents. In our tests, sunlight, nitrate, cellulose seals on acid stock bottles, and New York City tap water have given positive results. The cited authors [19,23,32] have selected wave lengths in the 500 to 600 mμ range and assumed that the color intensity is directly proportional to the amount of carbohydratelike material present.

Testing of each component of the media led to the development of MDV, which did not react with the reagent and, in fact, reflected the color (straw-yellow) of the *N*-ethyl carbazole. In tests of the algal cultures using MDV medium, accurate and reproducible results were unattainable, because of the differences in color reactions between the reference sugar solutions that were used to obtain standard curves, and the varied colors of our algal test solutions. It is apparent that the *N*-ethyl carbazole reagent was reacting with some unknown metabolic product of the organisms. Cultures were grown from small inocula and harvested during the late log phase to minimize any contribution from cell lysis. Cultures in the stationary or death phase had more "carbohydratelike" substances that were contributed, apparently, to the supernatant by lysis of spent cells. TABLE 4 shows some of the colors produced by cell-free supernatants of the algal cultures. *Katodinium* gave the only positive *N*-ethyl carbazole reaction typical of sugar; the other supernatants tested gave a positive color reaction, but one that differed from the control and *Katodinium.*

TABLE 4
TESTS OF CELL-FREE SUPERNATANTS FOR SUBSTANCES GIVING POSITIVE *N*-ETHYL CARBAZOLE TEST

Test supernatants	Color
MDV medium	straw-yellow
MDV + glucose	pink-purple
K. dorsalisulcum	purple
Melosira sp.	deep green
Gyrodinium sp.	straw-yellow
Gymnodinium sp.	light green
Monochrysis lutheri	straw-green
Prymnesium parvum	light green

DISCUSSION

The statement of K. M. Rae of the Scottish Marine Biological Association, Edinburgh, should serve as a guide in considering the presence of algae-derived organic material in the marine biotope: "We require, first, more realistic experiments in the laboratory designed to test the relative importance of various environmental factors in order to ensure that we are measuring those which are significant in the sea."[34]

Aside from the interest lent to any microbial product by its consideration in terms of comparative biochemistry, the *Katodinium* material may serve as a useful marine carbohydrate for enrichment cultures of characteristically marine bacteria. Comparative studies along these lines with agar, alginic acid, and laminarin would be of special value, especially if these favored the growth of bacteria with superior production of growth factors such as B_{12}, thiamine, and biotin. Such a finding would provide a convenient ecological explanation for the survival value of carbohydrate production. What food chains are involved with the mucus, and the position of such food chains in the ecosystem of the tropics are subjects that can, as yet, only be surmised.

The detection of such substances (exocrines), would a priori indicate a requirement for them in the complex ecosystem. Collier[35] has demonstrated the effect of carbohydrates on the siphoning rates of shellfish. Some carbohydrates initiating the feeding response are found as hydrolytic products of *Katodinium* polysaccharide. Belcher and Fogg[36] list several organic substances that are utilized by algae in respiration, a few which are also identifiable with the dinoflagellate mucus.

Such correlations as these hint at the many interesting relationships awaiting examination. As for the production of antimetabolites, Allen and Dawson[37] have demonstrated antibiotic production by tropical benthic marine algae.

Comprehensive studies with axenic techniques should provide us with many substances, the biological activity of which may serve to elucidate plant-animal interrelationships, and thus provide new tools for pharmacological studies on the cell and tissue level.

Acknowledgments

We are grateful to the directorates of the Lerner Marine Laboratory, Bimini, Bahamas, West Indies, and the Institute of Marine Biology, University of Puerto Rico, San Juan, P.R., for the use of their facilities. Our special thanks go to Luis R. Almodovar, of the latter institution, for his assistance in collecting specimens.

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STUDIES ON THE TOXIC PRINCIPLES FORMED BY THE CHRYSOMONAD *PRYMNESIUM PARVUM* CARTER*

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Prymnesium parvum, a phytoflagellate of the Chrysomonadineae widely distributed in sea water, rock pools, and estuaries (Carter, 1937; Droop, 1954), at times produces extensive blooms in sheltered brackish water lagoons (Liebert and Deerns, 1920; Otterstrøm and Steemann-Nielsen, 1939). This massive growth of *P. parvum* has often resulted in mass mortality of fish populations, due to a soluble toxin formed by the phytoflagellate. In Israel, where the phytoflagellate first appeared in 1947 and then quickly spread throughout the country, mass growths of Prymnesium have become a constant problem and danger in the fish-breeding industry. The future survival and expansion of carp-breeding in brackish water areas now largely depend on the successful control of *P. parvum*. Studies of ecology, factors determining toxin production, control measures, and nature of the toxins formed by the phytoflagellate are thus of great economic importance. Furthermore, the possibility of the spread of *P. parvum* to new, as yet uninfected regions, has been recently underlined by the appearance of blooms and fish mortalities in southeastern Europe (A. Valkanov, personal communication, 1960). With the present great increase of fish-breeding in brackish water ponds in Asian countries it is clear that *Prymnesium* infections may yet assume a much wider than local significance.

In addition to the economic aspect of the study of *Prymnesium*, this organism presents a good model for studies of toxin formation in algae. Its usefulness as a research tool rests on the facts that pure, axenic cultures have been obtained (Droop, 1954*a*; Reich and Kahn, 1954), and that its nutritional requirements are known and can be satisfied in a simple, fully-defined medium (Droop, 1954*b*; McLaughlin, 1956; Rotberg, 1958). The isolation of products is thus not beset by the difficulties encountered with some other algae, such as *Gymnodinium*, whose pure culture growth is difficult and with which toxin producing contaminants have been encountered (Myers *et al.*, 1959). A special property of *P. parvum*, its great sensitivity to lysis by ammonia or acetic acid, makes it a suitable model for study of the penetration of weak electrolytes in nucleated cells (Shilo and Shilo, 1953; 1955; 1960).

Biological Activities of Toxins Formed by P. parvum

The early studies of Otterstrøm and Steemann-Nielsen (1939), Reich and Aschner (1947), Shilo and Aschner (1953), and Shilo *et al.* (1953) were carried out with mixed cultures, but nevertheless strongly indicated that the phytoflagellate was the organism responsible for the ichthyotoxic activity. *Prymnesium*-containing cultures not only killed all tested species of fish, but also mollusks (Otterstrøm and Steemann-Nielsen, 1939) and tadpoles. Assay sys-

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tems based on fish and tadpoles have been established (Shilo and Aschner, 1953). The complete resistance of tadpoles after metamorphosis indicated a close connection between susceptibility to *Prymnesium* toxin and a functional gill-breathing apparatus.

With the isolation of *P. parvum* in pure culture (Droop, 1954*a*; Reich and Kahn, 1954) the connection between fish toxicity and the phytoflagellate was clearly established (Yariv, 1958). Later work extended the known spectrum of biological activity of culture supernatants to hemolysis of erythrocytes from various animal species (Yariv, 1958) and to lysis of normal and tumor cells (Shilo and Rosenberger, unpublished observations, 1960).

Among the sensitive tissue cells are lines of Ehrlich ascites cells (Landschütz strain), HeLa cells, human amnion cells (FL strain), and the Chang strain of liver cells. In all of these nucleated cells the active compound induces the formation of pseudopodialike plasma extrusions (FIGURES 1 and 2), followed by a complete breakdown of the osmotic barrier, as shown by the uptake of trypan blue.

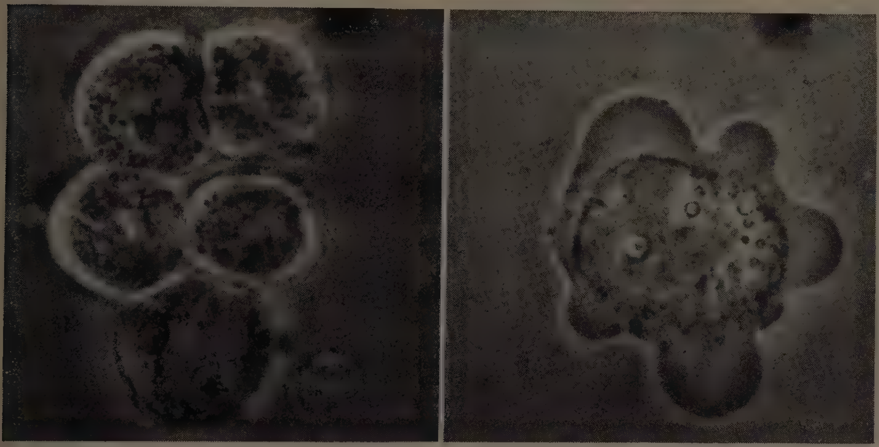

FIGURE 1. Lysis of human amnion cells (FL strain) by *Prymnesium* toxin. Photograph at left shows normal cells in phosphate-saline buffer plus 0.1 per cent methanol. The photograph at right shows lysing cells in contact with a 1:1000 dilution of a methanolic solution of *Prymnesium* toxins, with a hemolytic titer of 20,000 H.U. Photographs were made after 15-min. incubation at 37° C.

The range of activities shown by materials from pure cultures of *P. parvum* raises the question of whether a single toxic factor is responsible for all the observed activities, or whether many factors are involved. Comparison of the ratio of hemolytic to ichthyotoxic activity in various preparations from *P. parvum* cultures showed that this ratio may vary from >700 to 1. Thus besides ichthyotoxin, a hemolysin that is inactive against fish is formed by the phytoflagellate. This conclusion is further borne out by changes in the same preparation following selective absorption of the hemolysin by erythrocyte ghosts, or its destruction by alkaline methanol (TABLE 1). By such treatments the hemolytic titer may be reduced by 90 per cent without affecting fish toxicity.

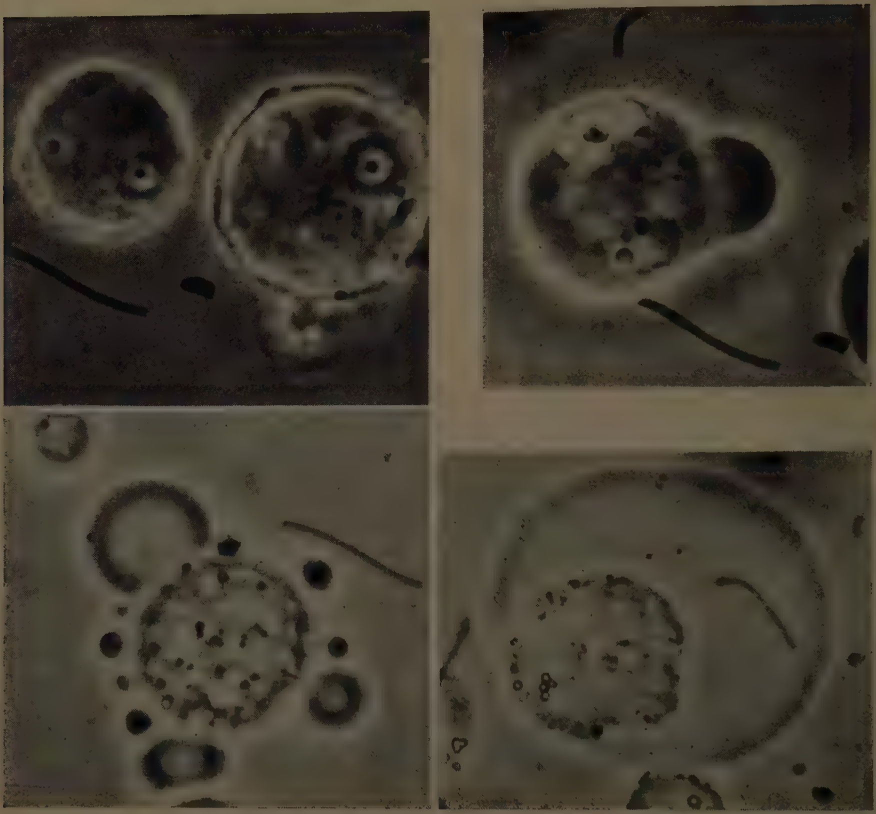

FIGURE 2. Lysis of Ehrlich ascites cells (Landschütz strain) by *Prymnesium* toxin. Normal cells are shown upper left. The other photographs indicate different stages of lysis under the conditions described for FIGURE 1.

TABLE 1

EFFECT OF 0.5 *N* NaOH HEMOLYTIC AND ICHTHYOTOXIC ACTIVITIES*

	Treatment	Ichthyotoxic units	Hemolytic units
Batch B 33	None	750	287,500
	+ alkali	600	7,200
Batch C 7A	None	800	160,000
	+ alkali	2,000	8,000

* The toxin was prepared by methanol extraction of dried *P. parvum* cells (Reich strain) harvested from a sea water-peptone-liver extract medium AB/8 (Droop, 1954; Yariv, 1958). Toxins were treated with alkali by dissolving solid NaOH in the methanol-toxin solution to give a final concentration of 0.5 *N* NaOH, and treatment was ended after 60 sec. by neutralization. Titrations for ichthyotoxic and hemolytic activity were carried out as described by Shilo and Aschner (1953) and Yariv (1958).

The possibility of the formation of several active components by *P. parvum* has been indicated by Yariv (1958), who achieved separation of two hemolytic fractions on paper chromatograms and of two active hemolytic and ichthyotoxic fractions in propanol-water systems. By chromatography on columns of Dowex-1 and stepwise elution with methanol-water mixtures, it has been possible to obtain further resolution of the hemolysins into 3 components (Rosenberger, unpublished observations, 1959).

In these separations, however, ichthyotoxin and activity against tumor and normal cells have not been examined, and their relations to the various hemolytic fractions remain to be clarified. In this connection, it is of interest that the conditions required for hemolytic and ichthyotoxic activity differ radically. Thus fish toxicity is manifested only at *p*H levels above 7, while hemolysis may be demonstrated at *p*H 5 and is lost at alkaline *p*H levels.

TABLE 2

RELEASE OF HEMOLYSIN FROM *PRYMNESIUM* UPON LYSIS*

Lysis	Hemolytic units	
	Cells	Supernatant
−	6875	—
+	5000	2000

* Cells were harvested from 100 cc. of a 2-week culture in AB/8 medium, incubation 28° C. (see TABLE 1), by centrifuging at 1000 rpm for 15 min. The packed cells were lysed by resuspension in 10 cc. of distilled water. Cells or cell debris were extracted for bound toxin with methanol. A similar number of cells extracted with methanol served for an estimation of total toxin. The determination of hemolytic activity against bovine erythrocytes was arried out as described by Yariv (1958).

Extracellular and Intracellular Occurrence of the Toxic Principles

The extracellular existence of *Prymnesium* toxins was first demonstrated by Otterstrøm and Steemann-Nielsen (1939), and has been fully confirmed by Reich and Aschner (1947) and by Shilo and Aschner (1953) for the ichthyotoxin, and shown by Yariv (1958) to apply also to the hemolysin. Neither the hemolysin nor the ichthyotoxin are, however, solely extracellular toxins, for they may be extracted from the cells in amounts larger than those present in supernatants (Rosenberger, *op. cit.*).

Lysis of cells by distilled water liberated hemolysin (TABLE 2) and repeated further extraction increased the yield. After no more hemolytic activity can be removed from cells by washing with distilled water, large amounts can still be recovered from these same cells by extraction with methanol (FIGURE 3). Water extraction yields approximately one fifth of the cell-bound hemolysin as measured by methanol treatment, and breaking of the cells in a mechanical disintegrator does not significantly increase the amount soluble in water. Ichthyotoxin again may be extracted from cells by methanol, but due to the much less sensitive and less accurate assay systems available, it is not yet clear what part of the cell-bound ichthyotoxin is extractable in water.

Since much greater amounts of hemolysin can be extracted from cells by methanol than by water, it may be that in the cell the active compound is present in a bound form, which is split by the organic solvent, similar to hemolysins found in animal tissues (Ponder, 1955).

An alternative explanation could be that free hemolysin in the cells is adsorbed by cell particles after lysis and re-eluted by the solvent. This would be consistent with the ready adsorption of *Prymnesium* hemolysin on various materials and its elution from them by methanol.

The high content of hemolysin and ichthyotoxin in cells has a bearing on control measures in the field, since sudden increases in the toxicity of the milieu may result from the application of substances causing lysis. Measurements of the concentration of cell-bound active materials could be, on the other hand, a useful means of following toxin synthesis in cultures and under field conditions. Extracellular hemolysin and ichthyotoxin are rapidly inactivated under growth conditions (FIGURE 4), while a greater stability could conceivably be postulated for the intracellular toxins.

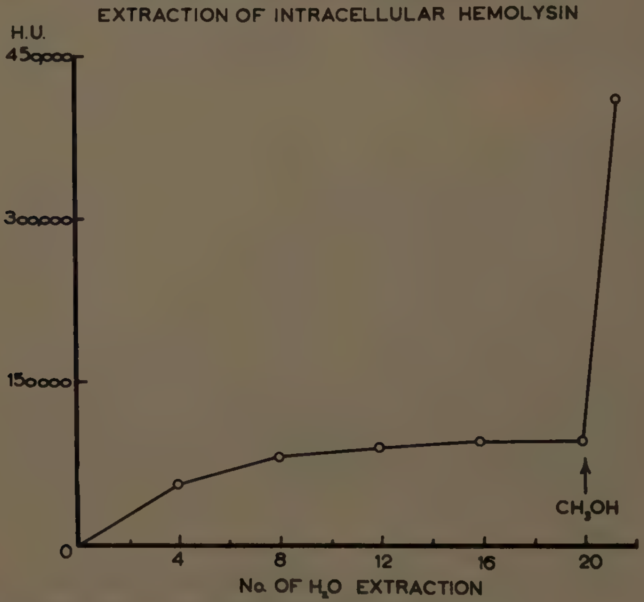

FIGURE 3. Using cells harvested from AB/8 medium (TABLE 1), 40.6 mg. of cells were extracted successively with 20 cc. of distilled H_2O by stirring for 10 min. at 4° C. Each group of four extracts was pooled and titrated. After 20 extractions the residual cell debris was extracted with methanol. The ordinate in this figure shows hemolytic units (H.U.) as defined by Yariv (1958).

Purification and Properties of the Toxic Principles

The first attempts to concentrate the hemolysins and ichthyotoxin from culture fluids and pond water, and to obtain them in a more easily handled and stable form, were made by Yariv (1958), using adsorption on $Mg(OH)_2$ precipitates as an initial step. Yariv obtained dry stable powders with an activity of up to 1300 hemolytic units (H.U.) and 400 ichthyotoxic units (I.U.) per milligram. Further purification was achieved by treatment with acetone and methanol, the purest materials having activities of 6700 H.U. and 1930 I.U. per mg.

With this method large quantities of liquid must be handled, and only the smaller part of the toxin, that present in the culture fluid, is obtained. These drawbacks may be largely overcome by using the cells as the source of toxin. After centrifugation, the cells are dried and extracted with acetone, in which the active materials are not soluble. The active compounds are then extracted with methanol; drying of these extracts yields residues with activities of up to 40,000 H.U./mg. The very high activity of these by no means pure materials is worthy of note, since in the same test system digitonin* has an activity of 800 H.U./mg., one fiftieth of that given by the *Prymnesium* hemolysin.

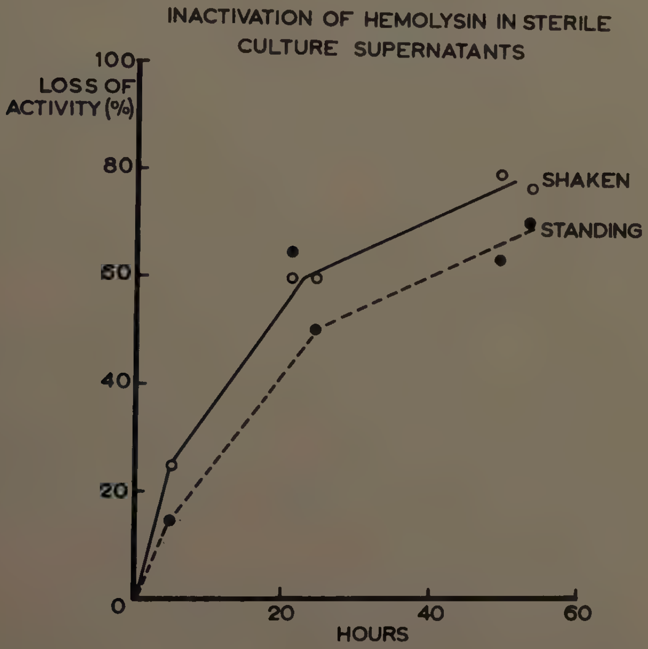

FIGURE 4. A culture in AB/4 (*p*H 7.5) was centrifuged, and the supernatants were stored at 27° C. under aseptic conditions. Shaking was done on a New Brunswick shaker, set at a slow speed. Hemolytic activity was estimated after prior acidification (Yariv, 1958).

The substances responsible for hemolytic and ichthyotoxic activity resemble each other in that they are soluble in methanol and insoluble in acetone, are nondialyzable, and are not retained by cation or anion exchange resins. Both ichthyotoxin and hemolysin have been found to be thermolabile under our conditions of test, being inactivated at a temperature of 70° C. or higher. Thus, although it is clear that *P. parvum* forms several hemolysins, at least some of which have no action on fish, all these active materials may well be closely related chemically.

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* C. P. Merck & Co., Inc., Rahway, N.J.

Exposure to *p*H levels above 5 for periods of several hours led to inactivation, which could be reversed by acidifying to a *p*H below 5. The rate and extent of this reversible inactivation varied from preparation to preparation (Yariv, 1958). Longer exposure to alkaline *p*H levels led to irreversible loss of activity. Exposure of hemolysin and ichthyotoxin to 0.5 *N* NaOH for short periods (Rosenberger, *op. cit.*) results in a 90 per cent reduction in hemolytic activity, marked (more than 75 per cent) loss of activity against Ehrlich ascites cells, but no reduction in the ichthyotoxic titer (TABLE 1). The inactivation at these relatively high concentrations of alkali is not reversed by acidification.

A special property of the ichthyotoxin is its requirement of cofactors for activity (Yariv and Hestrin, 1958). The toxicity of solutions has been shown by these workers to be a function of the concentration both of the fish toxin and of inorganic salts. Divalent cations were found to be more effective than monovalent ions, and streptomycin can also restore activity to dialyzed solutions of ichthyotoxin. A particularly interesting feature of this phenomenon is that while in the absence of inorganic salts or streptomycin the ichthyotoxin does not kill fish, it does sensitize them to later exposure to salts.

Speculations on the chemical nature of the active compounds obviously would be premature at this stage. While the nondialyzability of the active substances suggests a fairly large molecule, the mobility of the hemolysins on paper chromatograms developed with organic solvents militates against this, and these facts have led Yariv and Hestrin (1958) to speculate on the existence of a micellar form in aqueous solutions. Some resemblances between *Prymnesium* toxins and saponins have been pointed out by these workers. In this connection, the specific increase in nerve permeability to Ca ions, after treatment with saponin, is of interest (Valetter and Rossignol, 1951).

The Course of Toxin Formation

Shilo and Aschner (1953) demonstrated that there was no correlation between numbers of *P. parvum* in ponds or cultures and the amounts of ichthyotoxin present in the medium, and this observation has been confirmed by later workers for the ichthyotoxin and for the hemolysin (Yariv, 1958; Reich and Rotberg, 1958). However, all the above workers followed only extracellular toxin, and the data obtained are difficult to interpret because both the ichthyotoxin and hemolysin undergo rapid inactivation under field conditions (Shilo and Aschner, 1953) and even in bacteria-free laboratory cultures (FIGURE 4). Thus all that can be measured with the techniques at present available is the equilibrium between formation, release, and inactivation under the condition of test.

The accumulation of total toxin (intracellular plus extracellular) and, in particular, of total hemolysin, has been followed in growing cultures and in concentrated cell suspensions (Berman, 1960). In growing cultures, total hemolysin and ichthyotoxin and the content per cell of intracellular toxin increase with increasing age, the rise continuing in the stationary phase and in the phase of decline (FIGURE 5). In concentrated cell suspensions total hemolysin and intracellular hemolysin per cell increase during 3- to 4-day incubation periods (FIGURE 6), and these findings confirm the view that synthesis of hemolysin and ichthyotoxin are not processes closely associated with rapid multiplication of *P. parvum*.

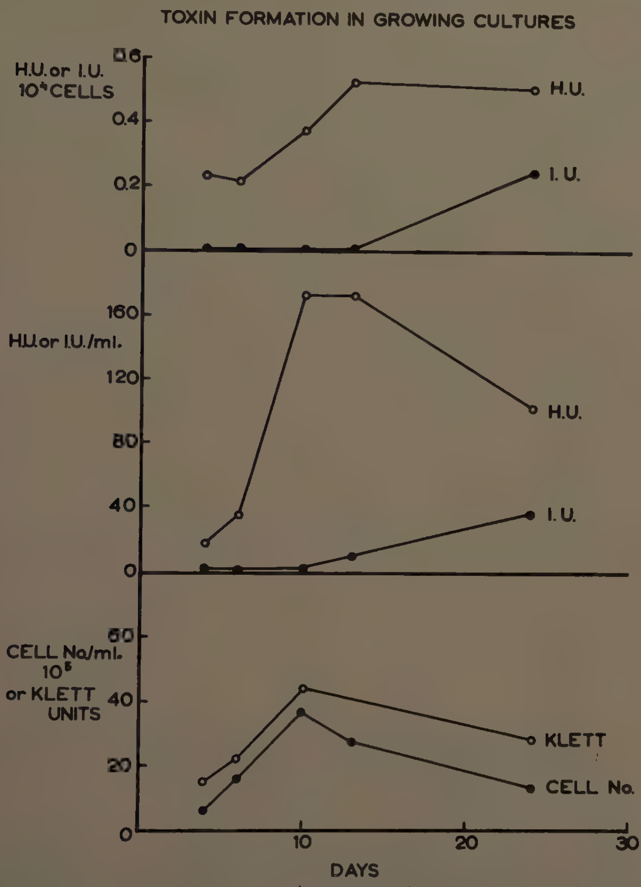

FIGURE 5. Cultures were grown in AB/8 medium (see TABLE 1) at 27° C. in continuous light. A series of 500-cc. Erlenmeyer flasks containing 100 cc. of medium were inoculated, and the entire contents of one flask was used for each examination. Cell counts (*lower*) were obtained in a hemocytometer after killing the organisms with I_2 vapor; turbidity was estimated in a Klett-Summerson photometer filter 66. Total (cell-bound + soluble) hemolysin (H.U.) and ichthyotoxin (I.U.) are plotted against time (*center*). Cell-bound toxin per 10^4 cells is presented (*upper*). For details of toxin estimations see TABLE 1.

Preliminary results of studies on the factors influencing hemolysin formation in concentrated suspensions show that this synthesis will proceed in a fully mineral solution not sufficient in itself to sustain growth (Berman, 1960). This further supports the hypothesis that growth and toxin formation are separable functions. Light has been found to be essential for hemolysin synthesis, as has already been suggested by Reich and Rotberg (1958) and Yariv (1958); the salinity of the medium has a profound influence on the production of hemolysin F and ichthyotoxin. In the case of the hemolysin, however, there appears to be an optimum concentration at which yields are at their highest (FIGURE 7).

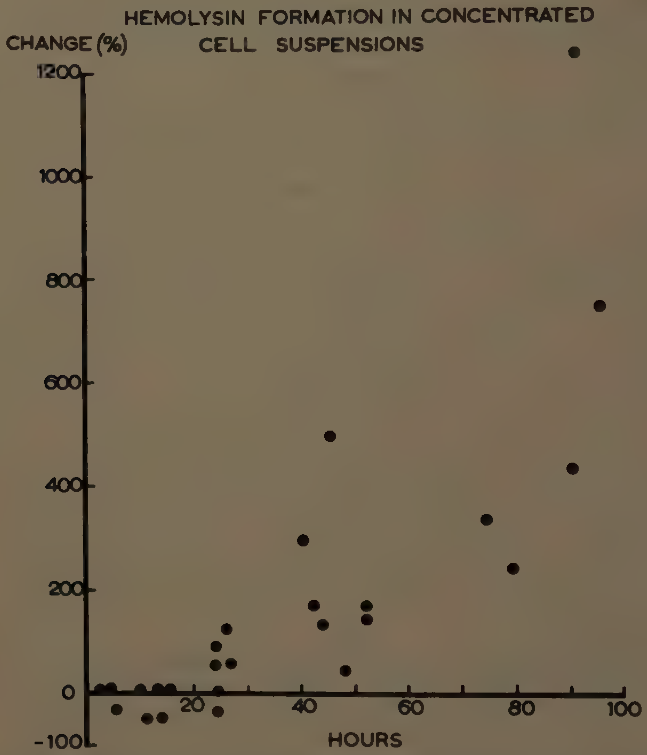

FIGURE 6. Cells grown in AB/4 were carefully washed and resuspended in the same medium to give an approximately 5-fold increase in cell concentration. Streptomycin (1 mg./cc.) was added and the suspensions incubated at 27° C. in continuous light. Cell-bound toxin was estimated and the percentage change of the initial titer was plotted against time.

Discussion

Comparison of the nature and properties of *Prymnesium* toxins to those of other algae shows some apparently unique features in the former. One of these is the dependence of ichthyotoxicity on the presence of inorganic salts or streptomycin. This phenomenon is an example of the complex interactions that may occur in natural habitats where several factors, in themselves without effect, synergistically produce far-reaching changes. Since *P. parvum* is an autotrophic organism, there would appear to be no obvious selective advantage in the production of hemolysins or ichthyotoxin. However the antibiotic effects of these on other competing microorganisms has not yet been explored. As in the case of the accumulation of antibiotics in soil, the concentration of the various toxins in natural habitats will be represented by a dynamic equilibrium between production and inactivation. In this instance, many of the factors involved in formation and destruction have been studied. On the physiological level, further investigation of the action of the ichthyotoxin may help to clarify the means by which entry of solutes is controlled in gill-breathing animals. While attack by *Prymnesium* toxins on a wide range of animal cells, including erythrocytes and various nucleated cells, is not a unique feature, the very high specific activity of these materials is striking.

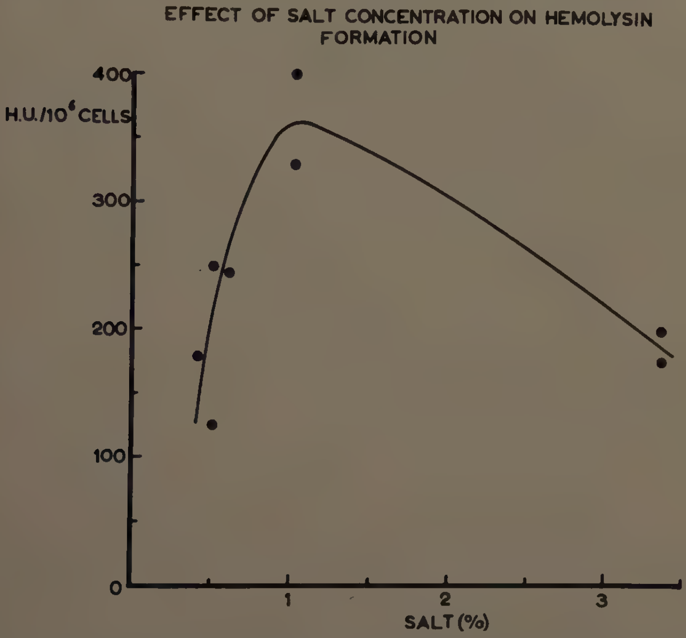

FIGURE 7. Concentrated cell suspensions were prepared as described in FIGURE 6. Osmotic pressure of the suspending medium was gradually and carefully changed by dialysis against a large volume of the same medium, containing various salt concentrations. Cell-bound hemolysin (H.U.) was estimated after 4 days incubation under the conditions described in FIGURE 6.

The solubility properties, nondialyzability in aqueous solutions, and chromatographic behavior of the active materials, show similarities with lipids rather than with other types of compounds. The possibility that in aqueous solutions the toxin may exist in micellar form has been stressed by Yariv and Hestrin (1958). Various treatments may thus affect the toxins by alterations in the physical properties of the aggregate, as well as by inducing chemical changes.

Acknowledgments

We are indebted to Shlomo Hestrin for his continued interest in this work, and to B. Golek for technical assistance.

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STEROIDS IN MARINE ORGANISMS

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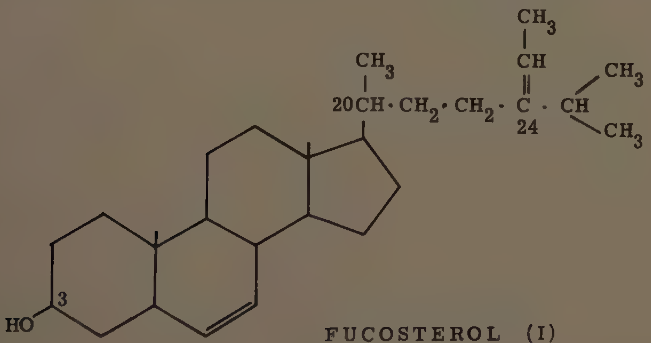
The steroids in marine organisms are of special interest because steroids are probably present in all truly cellular organisms, because it is thought that organic life began in the sea, and because evolution there has proceeded for an immense period in relatively stable conditions.

Sterols

Almost all, if not all cells apparently require sterols, which are perhaps essential for cellular structural integrity and organization. It appears that most Metazoa, except insects, can synthesize sterols from simple substances such as acetate, the process also involving an unsaturated aliphatic hydrocarbon, squalene $C_{30}H_{50}$, first isolated in 1906 from shark livers.

Marine algae are, in so far as is known, divided with respect to their sterols as follows: Phaeophyceae characteristically have fucosterol (I) $C_{29}H_{48}O$ (FIGURE 1), which also occurs in some Chlorophyceae and Rhodophyceae.[1,2] Sargasso weed (*Sargassum ringgoldianum*) has been shown[2] to contain the C-20 isomer of fucosterol (sargasterol). Fifteen species of Rhodophyceae have cholesterol (II), generally regarded as the characteristic animal sterol and not yet detected in any terrestrial plant.[3] Chlorophyceae possibly contain a sitosterol mixture of the kind found in green land plants.[1] With the methods used at the time,[1] sterols were not detected in three species of Myxophyceae, and it would be of great interest if further and more searching investigations confirmed this result. The pattern of sterol distribution strongly suggests different evolutionary histories for these types of algae and could perhaps provide clues as to which of them may have given rise to terrestrial plants.



FUCOSTEROL (I)

FIGURE 1.

The sterols of marine invertebrate animals have been extensively studied; a pioneer and much respected worker in this field was the late Werner Bergmann of Yale University, New Haven, Conn. Work on these substances is discussed elsewhere in this monograph.

There is no reason to suppose that marine vertebrates differ from terrestrial chordates in having cholesterol (and its C_{27} relatives) as their chief or, indeed, their only sterol. This question ought, perhaps, to be further explored, especially as it has been satisfactorily shown[4] that at least two (toad) species of the genus *Bufo* contain a significant proportion of what appears to be a C_{28} or C_{29} sitosterol mixture.

It seems that as evolution of Metazoa proceeded, sterols became converted into other steroids having diverse kinds of physiological activity; such substances are the steroid hormones, bile salts, certain poisons, saponins, and other compounds.

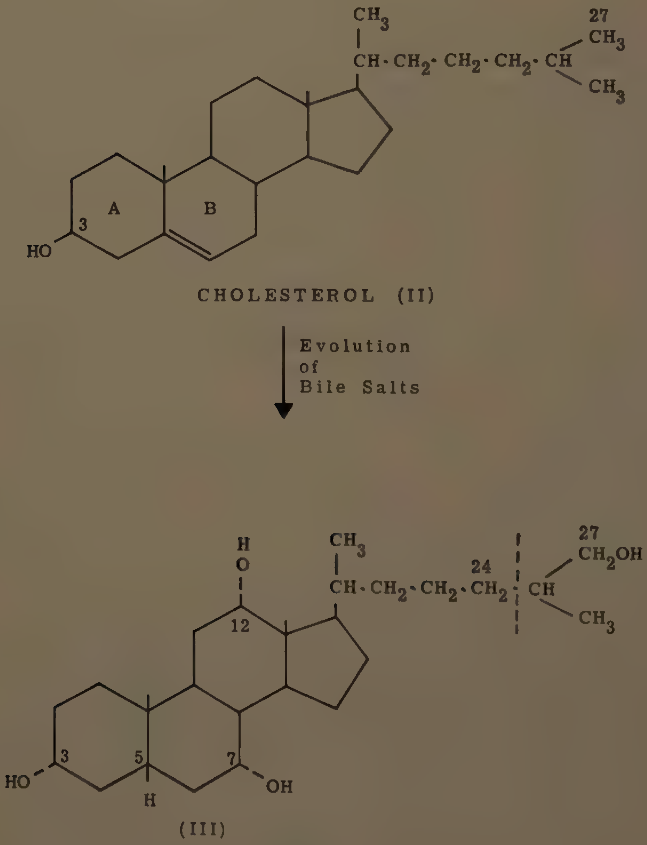
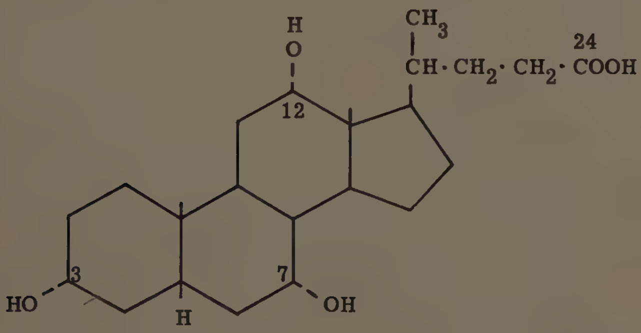


Figure 2.

Steroid Hormones

Little is known about the chemistry of steroid hormones of any but the common land animals; however, Wotiz *et al.*[5] have obtained evidence that "mammalian" estradiol (estradiol-17β) is present in the ova of the dogfish *Squalus suckleyi*. It is to be hoped that work of this kind will continue and be intensified, for it would be most interesting to know at what stage in evolution steroid hormones were derived from sterols. The well-known fact that some land plants contain steroid estrogens,[6] which it is difficult to believe could function estrogenically in such a situation, suggests that possibly a capacity to degrade sterols (or to divert sterol synthesis) to the C_{18}, C_{19}, and C_{21} types of steroid hormones may be, for some reason, widespread in living cells. If such degradations (or anomalous syntheses) occurred in primitive animals, the physiological properties of the resulting products might then have conferred selection advantages that would account for the persistence and diversity of such hormones in more advanced animal types. A consideration of the nature of marine environments, as indicated above, suggests that a search for steroid hormones amongst primitive sea animals ought to be undertaken.



CHOLIC ACID (IV)

FIGURE 3.

Steroids of Poisonous or Hemolytic Types

Many land plants and some animals (for example, toads) have evolved steroids of poisonous or hemolytic types; such substances are steroid alkaloids, saponins, and heart poisons. Substances of this kind are not yet well known in marine organisms, but J. D. Chanley, R. F. Nigrelli, Harry Sobotka and their colleagues have obtained saponinlike steroids from a holothurian, and these are discussed elsewhere in this monograph.

Bile Salts

The conversion of sterols into bile salts occurs in all vertebrates and has been suggested in at least some marine Crustacea.[7] The process cholesterol → bile acids has been directly studied in a number of land vertebrates.[8] In the rat, and probably in all modern species, it occurs in the liver and apparently proceeds in stages, as follows:

(1) Cholesterol (II) (FIGURE 2) molecules are hydroxylated and hydrogenated to give polyhydric alcohols of the A/B *cis* type, which have a terminal —CH_2OH group. An example is III (FIGURE 2) which, although it has not been isolated as such from biles, would be expected to be an efficient bile salt precursor in the species mentioned above.

(2) Alcohols such as III are converted to C_{27} carboxylic acids in which $—\overset{27}{C}H_2OH$ (see III) becomes $—\overset{27}{C}OOH$.

(3) β-oxidation at C-24 gives the C_{24} bile acids, of which cholic acid (IV) (FIGURE 3) is the commonest example. The above stages (1) to (3) appear, in a sense, to have become "frozen" in some species during evolution; that is, there are species that cannot proceed beyond stages (1) or (2) and have in their bile alcohols such as III (conjugated with sulfate) or the corresponding carboxylic acids (conjugated with taurine). On the other hand, most existing species that can be described as "modern" do degrade cholesterol fully to cholic and similar bile acids.

An unexplained feature is that some species, or groups of species, have chemically unique bile salts. The "reasons" for this are perhaps lost in the past, but the distribution of such substances may be expected, eventually, to throw light on evolutionary history.

Different types of marine vertebrates will now be referred to and the nature of their bile salts discussed in the light of the considerations set out above.

Actinistia. The only known living representative of this ancient order, the coelacanth *Latimeria chalumnae* Smith, has bile salts of the alcohol-sulfate type, and not, as far as is known, like those of any other animal.[9] Further chemical examination may modify this conclusion, which is, perhaps, an expected one in view of the unique and primitive nature of *Latimeria.*

Selachii. About seven species of sharks and two species of rays have been examined, and from the bile of each has been isolated scymnol, an alcohol of probable formula $C_{27}H_{46}O_5$, and very similar to (III) in chemical constitution.[6] Although further chemical work is needed on the exact molecular structure of scymnol, as well as on the question of whether it actually occurs as a sulfate in the bile, there is no doubt that a characteristic constituent of selachian bile is an alcohol of this type. This alcohol has been found, as yet, nowhere else in nature, and its apparently universal derivation from the bile of Selachii emphasizes the isolated position of this group of animals. More work is needed on the distribution of scymnol (or its chemical precursor) in these fishes, and especially on the chemical nature of the acids that their bile also contains. It would be particularly interesting to know whether there are signs of the evolution of bile acids of a modern type; experiments designed to investigate this point would have to exclude the bile acids that will normally enter selachian gall bladders from the animals' food.

Teleostei. Examination of the bile salts of about 30 species in this class has revealed only "modern" C_{24} bile acids, such as cholic acid (IV), conjugated with taurine. An early claim[10] was that these conjugates, unlike the bile salts of land animals and fresh-water fish, existed chiefly as potassium (rather than sodium) salts; this was later denied.[11] It might still prove interesting to compare the biliary excretion of Na^+ and K^+ of marine with that of fresh-water and terrestrial forms. The C_{24} bile acids are those generally found in snakes, birds, and mammals, and their presence in marine teleosts strongly argues that separate evolution of bile salts, through the stages (1) to (3) above, must have occurred in the sea. The alternative view, that snakes, birds, and mammals all descended from modern marine teleosts, seems untenable.

In unpublished work I have examined the bile of three species of sturgeons (*Acipenseridae*) and have found that it contains chiefly taurine conjugates of cholic acid. However, after hydrolysis a small proportion of neutral material was found, and this will be further investigated to see whether it is a sign of the "primitive" characters that sturgeons' bile might have been expected to show. The absence of these characters argues against the primitive nature of the phenotype, by analogy with other vertebrate examples. A bile acid (tetrahydroxynorsterocholanic acid) isolated by Ohta in 1939[16] from certain marine and fresh-water teleosts was formerly thought to have the formula $C_{27}H_{46}O_5$, and hence to be of a (chemically) "primitive" type. It has recently been proved[12] to be *allo* (5α) cholic acid, $C_{24}H_{40}O_5$. Its biological significance is uncertain. I have detected it in sturgeons' bile, where, however, it cannot necessarily be regarded as throwing light on the history of these fishes.

It is especially interesting that marine teleosts do not, apparently, contain cyprinol, the bile alcohol found[13] in all fishes so far examined of the family Cyprinidae (order Ostariophysi). The Ostariophysi are thought to have evolved separately in fresh water, and perhaps they have retained cyprinol as a "memento" of a remote marine ancestor. The chief chemical features of cyprinol are also found in a bile alcohol from some frogs (Ranidae), and it should be kept in mind that such features might be detectable in primitive marine forms, which could then be regarded as indicators of the ancestry of the Ostariophysi.

It is obvious that there is ample scope for a much wider survey of marine fish bile, and it is important to include in this examples of Petromyzonidae, Myxinidae, and Chimaeridae, for these might well give bile salt clues to evolutionary history.

Chelonia. Only one truly marine species, the green turtle, *Chelonia mydas*, of this order has recieved a preliminary investigation, and in only two species in all have bile salts been chemically identified. In these two cases, C_{27} acids were found, suggesting that chelonian bile is "primitive" in chemical type.[14]

Crocodilia. These survivors of the Archosauria have few sea-going species, the bile of none of which has been examined. Three other species all have C_{27} acids of probably essentially the same chemical types as those of the Chelonia.[14]

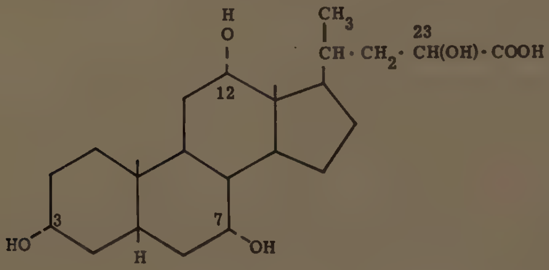
Cetacea. Three species of whales contain bile salts indistinguishable from those of other modern Eutheria.[14] Glycine, as well as taurine, conjugation is present; there is circumstantial evidence that this is an especially modern character. There are thus, as far as is known, no features of cetacean bile salts that would suggest any special influence of their marine life, or give any indication of their history.

Pinnepedia. Seals and walruses (ten species of three families) contain cholic acid and phocaecholic acid, together with a bile acid, 12α-hydroxy-phocaecholic acid (23-hydroxycholic acid, V), as shown in FIGURE 4, found so far in only one other group in the animal kingdom.[14] This group is of snakes of the genus *Bitis*, which agree with the Pinnepedia in having C-23 hydroxylated bile acids.

It is not definitely known whether the bile of Pinnepedia contains glycine conjugates and this is a point worth elucidation in the reasonable hope that the result might eventually contribute toward an understanding of the position of the order among Mammalia. All this apart, the chemical study of Pinnepedia bile salts supports the remarks of Scheffer[15] that the order is "isolated, aberrant, unique," and also that it is of monophylogenetic origin. The bile salt chemical findings do not support the views of those, quoted by Scheffer who, on other chemical grounds, stress the resemblance between the Pinnepedia and other modern Carnivora. In this respect there is a clear difference between bile salts of Cetacea and those of Pinnepedia.

No other marine mammals have been examined. The definite biological results obtained in the study of Pinnepedia bile encourage hope that an investigation of the Sirenia might give valuable information about the relationships of these animals.

The detailed chemical examination of bile salts is not particularly easy and it certainly requires experience. However, useful and sometimes sufficient information may be obtained by such simple methods as paper chromatography. There does not seem to be any practical reason why the method of bile salt comparisons should not be much more widely applied to biological problems, especially in marine vertebrates, than has hitherto been the case.



12α - HYDROXY-PHOCAECHOLIC ACID
(V)

FIGURE 4.

The question of how far gall bladder bile may be affected by products of microbial action in the intestine during the enterohepatic circulation has been explored in some of the terrestrial species mentioned above.[8] Such microbial action has produced some striking results, especially in the apparent reactions of the animal to the types of bile salt imposed on it by its intestinal parasites. However, none of the effects detected seems likely to invalidate the biological conclusions set out above.

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EFFECTS OF HOLOTHURIN, A STEROID SAPONIN FROM THE BAHAMIAN SEA CUCUMBER (*ACTINOPYGA AGASSIZI*), ON VARIOUS BIOLOGICAL SYSTEMS*

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Introduction

The toxicity of certain sea cucumbers to fish has been known since the latter part of the last century (Hyman, 1955). In Guam and in other regions of the Indo-Pacific, the juices of some species, for example, *Holothuria argus* and *H. atra*, are used by natives for poisoning pools among coral reefs as an aid in catching fish for food (Frey, 1951). Thirty toxic species, belonging to four of the five orders of Holothuroidea, have thus far been reported (TABLE 1); nothing is known about the possible toxic effects of the deep-sea inhabiting Elasipoda. However, it is highly probable that there are more toxic species than the list indicates and, possibly, they are more widely distributed.

Thelenota ananas, *Stichopus variegatus*, *Holothuria atra*, *and H. axiologa* are toxic sea cucumbers highly valued as food and prepared for the Oriental market as *trêpang* (Yamanouchi, 1955; Hyman, 1955). The toxicity is lost or considerably reduced by repeated boiling and/or by the digestive action of enzymes (Frankel and Jellinek, 1927; Yamanouchi, 1955). There is also considerable variation in the degree of toxicity among the species analyzed; nothing is known about seasonal differences.

Holothurin

The active principle or principles in the Bahamian sea cucumber (FIGURE 1) is concentrated in the Cuvierian tubules,† and was named holothurin by Nigrelli in 1952.‡ The tubules, attached to the common stem of the respiratory organs near the region where the intestinal tract enters the cloaca, are reddish, branching filaments that contain structures filled with granules (FIGURE 2); the granules are apparently the source of holothurin.

Sun- or oven-dried tubules are referred to as crude holothurin, and preliminary analysis shows the following constituents: glycosides and pigment (60 per cent), cholesterol (1 per cent), insoluble proteins (5 to 10 per cent), salts, polypeptides, and free amino acids (30 per cent). By paper chromatography the following free amino acids were identified: alanine, arginine, cystine, glycine, glutamic acid, histidine, serine (or lysine), and valine. In the studies presented here both the fresh and sun-dried crude material was used in aqueous solutions; some comparison is made with the cholesterol-precipitated fraction designated as holothurin A, which represents 40 per cent of the crude holothurin. Holo-

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* The work described in this paper was supported in part by Contract NONR 2266(00) from the Office of Naval Research, Department of the Navy, Washington, D. C.

† Cuvierian tubules are absent in some toxic holothurians and devoid of the poison in other species. The active substance is extracted from the body wall with hot ethanol.

‡ The name holothurin was also used by Yamanouchi (1955) for the ethanol extract of the body wall of *Holothuria vagabunda*.

thurin A has been partially characterized chemically and given the empirical formula $C_{50-52} H_{81-85} O_{25-26} SNa$ (Chanley *et al.*, 1959, and elsewhere in these pages). This material resembles digitonin and other saponins in forming a complex with cholesterol.

TABLE 1
HOLOTHURIANS TOXIC TO FISH

Species	Habitat	Author
Dendrochirota		
Pseudocucumis africana	Seto, Japan	Yamanouchi, 1955
Cucumaria japonica	Onagawa, Japan	Yamanouchi, 1955
Cucumaria echinata	Onagawa, Japan	Yamanouchi, 1955
Cucumaria sp.	Onagawa, Japan	Yamanouchi, 1955
Pentacta australis	Onagawa, Japan	Yamanouchi, 1955
Aspidochirota		
Holothuria argus	Torres Strait Region, Pacific	Clark, 1921
	Palau Island, Pacific	Yamanouchi, 1955
Holothuria atra	Guam, Pacific	Frey, 1951
	Palau Island, Pacific	Yamanouchi, 1955
Holothuria impatiens	Bimini, Bahamas	Nigrelli
	Mediterranean	Arvy, 1954
Holothuria tubulosa	Mediterranean	Arvy, 1954
Holothuria polii	Mediterranean	Arvy, 1954
Holothuria vagabunda	Seto, Japan	Yamanouchi, 1955
Holothuria monocaria	Seto, Japan	Yamanouchi, 1955
Holothuria lubrica	Seto, Japan	Yamanouchi, 1955
Holothuria scabra	Palau Island, Pacific	Yamanouchi, 1955
Holothuria bivittata	Palau Island, Pacific	Yamanouchi, 1955
Holothuria lecanora var.	Palau Island, Pacific	Yamanouchi, 1955
Holothuria nobilis	Palau Island, Pacific	Yamanouchi, 1955
Holothuria axiologa	Palau Island, Pacific	Yamanouchi, 1955
Holothuria sp.	Palau Island, Pacific	Yamanouchi, 1955
Actinopyga agassizi	Bimini, Bahamas	Nigrelli, 1952
Stichopus bandiotus	Bimini, Bahamas	Nigrelli, 1952
Stichopus japonicus	Seto, Japan	Yamanouchi, 1955
Stichopus variegatus	Palau Island, Pacific	Yamanouchi, 1955
Stichopus chloronotus	Palau Island, Pacific	Yamanouchi, 1955
Parastichopus nigripunctatus	Japan Sea	Yamanouchi, 1955
Thelenota ananas	Palau Island, Pacific	Yamanouchi, 1955
Elasipoda	(None)	
Malpadonia		
Caudina chilensis	Asamushi, Japan	Yamanouchi, 1955
Apoda		
Euapta lappa	Bimini, Bahamas	Nigrelli
Leptosynapta ooplax	Seto, Japan	Yamanouchi, 1955
Polycheira rufescens	Seto, Japan	Yamanouchi, 1955

Holothurin and Evisceration

The Bahamian sea cucumber, as other holothurians, undergoes evisceration and autotomy when disturbed. These reactions can be induced by excessive handling, in or out of water, or by exposing the animals to high temperatures or to oxygen-deficient conditions. The process of evisceration follows a definite pattern. It is initiated by a vigorous contraction of the body wall, followed by a slow extrusion of the Cuvierian tubules through a rupture in the cloacal wall and, finally, by a sudden, almost explosive, expulsion of the in-

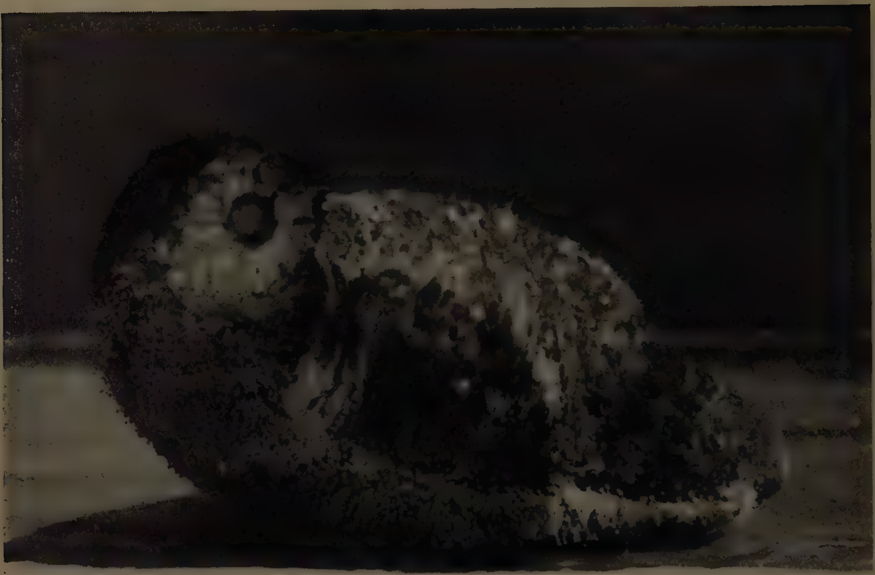

Figure 1. The Bahamian sea cucumber *Actinopyga agassizi* Selenka, shown one half natural size.

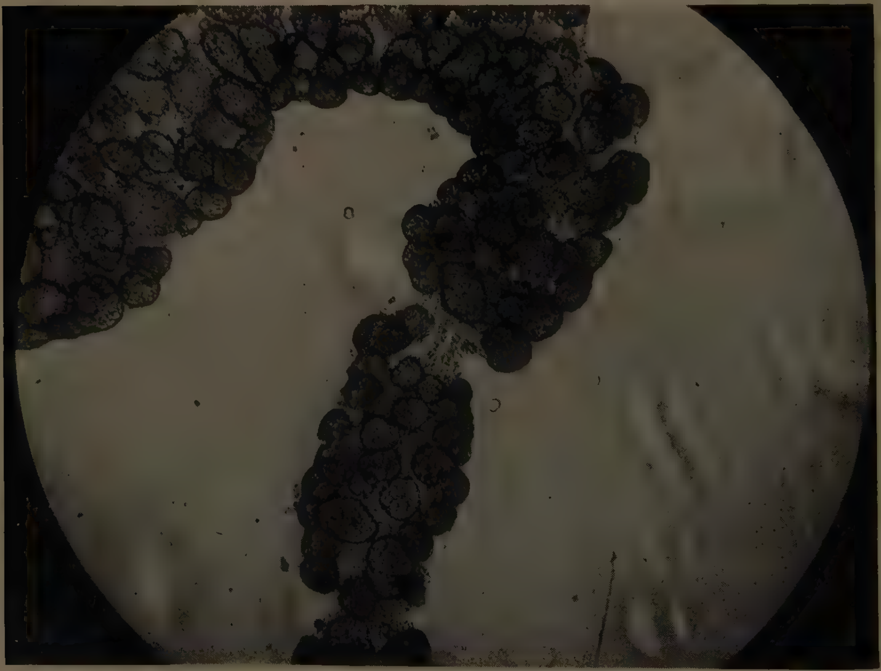

Figure 2. A single filament of the Cuvierian tubules. Structures filled with granules are probably the source of holothurin.

testinal tract and genital glands out through the anus. Autotomy occurs when these organs break off from the rest of the body. The structures remaining within the animal are remnants of mesenteric tissue, the cloaca, respiratory organs, the anterior tentacles, and all parts of the water vascular system. In time, the eviscerated and autotomized parts are regenerated (Hyman, 1955; Mosher, 1956).

The exact relationship of the poison-laden Cuvierian tubules to the phenomenon of evisceration is not definitely known, although the evidence seems to indicate that they are intimately associated. Injection of holothurin solution made from fresh tubules will induce evisceration as will the introduction of this solution or of the tubules into the water in which intact animals are kept; the more poison that is present the quicker the reaction. It should be pointed out that such eviscerated animals remain toxic to fish for several days since some of the poison is absorbed by the skin and respiratory organs; none of the poison is present in the intestine and genital glands* and in the fluid of the Polian vesicles, structures acting as expansion chambers for the water vascular system.

Effects of Holothurin on Fishes and Invertebrates

The so-called "pearl" fish, *Carapus bermudensis*, that lives as an inquiline in the cloacal and respiratory cavities of *Actinopyga agassizi*,† is susceptible to the toxic action of holothurin, as are other fishes that live in the surrounding environment. The lethal concentrations varied from 100 ppm in 3 to 15 min. to 1 ppm, or less, in 4 to 8 hours, at 25 to 30° C. The reactions were characterized by great irritability and gasping movements, followed by loss of equilibrium and power of locomotion with bleeding at the gills. No recovery occurred when these symptoms appeared.

Toxicity tests were also made on crustaceans, mollusks, annelids, coelenterates, and on a number of echinoderms, including other holothurians all living in the same environment. Suffice to say that the animals tested exhibited a variety of reactions, ranging from simple irritability to death, when placed in the same tank with one or more sea cucumbers that had eviscerated. The concentration of the poison under these conditions is extremely high.

Effects of Holothurin on Development of Sea Urchin Eggs

In order to evaluate the possible effects of extreme dilutions of holothurin on plankton life, toxicity studies were made on various stages of development of the sea urchin, using as the test animal the northern species *Arbacia punctulata*, for which the developmental changes under specified conditions have been well characterized (Harvey, 1956). Pre- and postfertilized eggs at various stages of development were treated with crude holothurin and holothurin A solutions for 5 min., or immersed continuously in concentrations varying from

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* The intestine of *Stichopus variegatus* and the testes of *Holothuria scabra*, toxic species from Palau Is., are eaten raw by natives (Yamanouchi, 1955).

† The "pearl" fish can be removed safely from its host. This is done by cutting open freshly collected sea cucumbers before the Cuvierian tubules are extruded into the cloaca, and quickly transferring the fish to clean uncontaminated sea water. Under such conditions the fish have been kept alive for indefinite periods of time and used for evaluating the toxicity of holothurin, and for other experimental purposes (Aronson and Mosher, 1951).

1000 ppm to 0.001 ppm (Ruggieri and Nigrelli, 1960). Both substances produced similar developmental faults, characterized chiefly by animalization, a process in which gastrulation fails and in which there is also a hyper development of ciliary tuft and a thickened apical plate. Such abnormalities are supposedly due to specific action on the protein structures of the cell, and opposed to vegetalization in which carbohydrate metabolism is affected. Other abnormalities produced were inhibition of hatching, fragmentation, radialization, and defective development of skeletal structures and arms in the fully formed plutei. Continuous immersion and 5-min. exposures of eggs in solutions of 0.01 ppm and 1 ppm resulted in such abnormalities (TABLE 2).

TABLE 2
EFFECTS OF HOLOTHURIN ON DEVELOPMENT OF *ARBACIA**
Continuous and 5-min. Exposure

Concentration (ppm)	Prefertilization				Postfertilization			
	Holothurin	Saponin	Holothurin	Saponin	Holothurin	Saponin	Holothurin	Saponin
	5-min.		Continuous		5-min.		Continuous	
	(%)	(%)	(%)	(%)	(%)	(%)	(%)	(%)
100	0	85 *P*	0	0	0	85 *P*	0	0
10	0	95 *P*	0	85 *pP*	1–10 *a*	95 *P*	0	85 *Pp*
5	40 *a*	95 *P*	—	—	85 *ar*	95 *P*	—	—
1	85 *arp*	—	0	95 *P*	95 *praP*	—	0	95 *P*
0.1	95 *Pp*	—	85 *ap*	95 *P*	95 *Pp*	—	50 *a*	95 *P*
0.05	95 *P*	—	—	—	—	—	—	—
0.01	—	—	95 *prai*	—	95 *P*	—	95 *praP*	—
0.001	—	—	95 *P*	—	—	—	95 *P*	—

* *P* = normal plutei; *p* = abnormal plutei; *a* = animalization; *r* = radialization; and *i* = inhibition of hatching.

Toxicological and Pharmacological Effects of Holothurin on Nonmarine Organisms

Lethal and other effects on plants and animals. Our studies on the effects of crude holothurin on nonmarine organisms in concentrations of 1000 ppm showed that, in addition to being lethal, it also caused suppression of root hair development in water cress and necrosis of onion root tips; it had been determined earlier that it caused retardation of pupation of fruit fly (Goldsmith *et al.*, 1958), in the same concentration. The lethal concentrations for a number of other nonmarine organisms are shown in TABLE 3. Approximately similar levels of toxicity were obtained by Yamanouchi (1955) for the "holothurin" extracted from the body wall of *Holothuria vagabunda* Selenka, a species commonly found in Japanese waters.

Neurotoxic properties of holothurin A. It is evident from observations on fish that crude holothurin has some neurological properties. This fact is substantiated by the results obtained by Friess *et al.* (1959), who showed that holothurin A is a potent neurotoxic agent and is comparable to cocaine, procaine, and physostigmine in potency when tested on desheathed bullfrog sciatic nerve and rat phrenic nerve-diaphram preparations. Furthermore, holothurin A has a direct contractural effect on the muscle. The pharmacodynamic action of this substance is reported elsewhere in this monograph by Friess *et al.* and will not be discussed here except to say that the action of holothurin A on such preparations is irreversible.

Hemolytic and other hematological effects of crude holothurin. The hemolytic properties of holothurin were tested on rabbit red blood cells and compared with saponin.* A 10 per cent solution of heparin and warfarin and a 5 per cent solution of dehydrocholic acid were used as controls and found to be nonhemolytic. The rabbit blood was collected in 0.1 *M* calcium oxalate, centrifuged, and washed with 0.9 per cent saline until the supernatant was clear. Solutions of holothurin and saponin were prepared in saline and in saline-cholesterol; dilutions of each were made in concentrations ranging from 200 ppm to 30 ppm. One cc. of each dilution was placed in hematological test tubes and 0.1 cc. of a 2 per cent red cell suspension was then added to each. The results, given in TABLE 4, show that holothurin is slightly more effective

TABLE 3
TOXIC EFFECTS OF CRUDE HOLOTHURIN ON SOME NONMARINE ORGANISMS

Organism	Holothurin (ppm)	Effects	Author
Protozoa			
Ochromonas malhamensis	22	Inhibition of growth	Nigrelli & Zahl, 1952
Euglena gracilis	66	Inhibition of growth	Nigrelli & Zahl, 1952
Amoeba proteus	100	Lethal	Present paper
Tetrahymena pyriformis	22	Inhibition of growth	Nigrelli & Zahl, 1952
Paramecium caudatum	10	Lethal	Present paper
Coelenterates			
Hydra (brown)	10	Lethal	Present paper
Nemathelminthes			
Nematodes (free-living)	100	Lethal	Present paper
Mollusca			
Planorbis sp.	1–10	Lethal	Present paper
Annelida			
Tubifex tubifex	10	Lethal	Present paper

TABLE 4
HEMOLYTIC EFFECTS OF HOLOTHURIN ON RED BLOOD CELLS FROM RABBIT

Concentration (%)	Holothurin		Saponin	
	Saline (%)	Saline-cholesterol (% hemolysis)	Saline (%)	Saline-cholesterol (% hemolysis)
0.02	100	50–100	100	50–100
0.01	100	0–50	100	0–50
0.008	50–100	0–20	50	0
0.004	50	0	0	0
0.003	0	0	0	0

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* These experiments were carried out by Steven Carson.

as a hemolytic agent than is the reference substance saponin. The data also support the saponinlike character of holothurin, especially in its loss of potency when mixed with cholesterol.

It was also found that 0.2 ml. of a 5 per cent solution of crude holothurin injected in the dorsal lymph spaces of winterized frogs (*Rana pipiens*) not only produced 50 per cent hemolysis, but also stimulated hemopoiesis in the bone marrow (Jakowska *et al.*, 1958). This stimulation was unexpected, since previous studies by Jordan and Speidel (1923) showed that this hemopoietic structure remained refractory in winterized frogs when treated with saponin, or following hemorrhages and splenectomy, which under other conditions would activate hemopoiesis. It is believed that the activation of the bone marrow by holothurin is due to factors other than hemolysis.

Effects of holothurin on regeneration of planarians. Some interesting results were obtained when planarians were treated with holothurin (Quaglio *et al.*, 1957). Animals immersed in a solution of 100 ppm for 5, 10, and 15 min. disintegrated when returned to clean water; those treated for 30 and 45 sec. and for 1 min. survived. When the latter animals were cut transversely, the anterior portions, known to exhibit a higher metabolism, regenerated into complete animals; the posterior segments, with lower metabolic gradient, disintegrated or failed to regenerate. These results are further indications of the possible antimetabolic activity of holothurin.

TABLE 5

COMPARISON OF THE EFFECTS OF CRUDE AND PURIFIED HOLOTHURIN ON KREBS-2 ASCITES TUMORS IN FEMALE SWISS MICE

Animals (No.)	No. of tumor cells ($\times 10^6$)	Holothurin (mg.)	Mean survival (days)	Mean weight on 10th day (gm.)
With crude holothurin				
186	2	—	9.3–15.1	42.0
24	2	0.10*	25.4†	23.5
With purified holothurin				
18	3	1.0–1.5	14.0	31.5

* Ten injections in twenty days.

† Some of these mice lived as long as 18 months.

Antitumor properties of crude holothurin. It was shown early in these studies that solutions of crude holothurin were capable of suppressing the growth of Sarcoma-180 (Nigrelli, 1952; Nigrelli and Zahl, 1952). A similar effect was demonstrated for Krebs-2 ascites tumors (Sullivan *et al.*, 1955; Sullivan and Nigrelli, 1955). Some of the results are summarized in TABLE 5. As may be seen, 0.1 mg. of crude holothurin injected intraperitoneally into ascites-bearing mice every other day for 20 days produced a marked reduction in mean body weight on the 10th day and an increase in survival time (up to 18 months). The lethal dose of crude holothurin for these animals is 0.2 mg. when given intraperitoneally in a single injection. However, the antitumorous principle apparently is lost or considerably reduced in the purification process. One sample of processed holothurin showed a 10-fold loss in toxicity and no antitumor effect. The lethal dose for this sample was 2 mg. for 22-gm. female Swiss mice.

Discussion

It is evident that crude holothurin from the Bahamian sea cucumber *Actinopyga agassizi* exhibits a variety of effects on biological systems. Outstanding features are the rapidity with which the effects are produced even in extreme dilutions, its ability to combine with various components of the cell, and its relative irreversibility of action. The actions on the development of sea urchin eggs, regeneration of planaria, hemopoiesis in bone marrow of winterized frogs, and inhibition of tumors in mice suggest that holothurin may act as an antimetabolite.

Acknowledgment

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SOME PHARMACOLOGIC PROPERTIES OF HOLOTHURIN A, A GLYCOSIDIC MIXTURE FROM THE SEA CUCUMBER*

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Introduction

Interest in the toxic principle holothurin, derived from the sea cucumber *Actinopyga agassizi* Selenka, stems largely from recent observations (Nigrelli, 1952; Nigrelli and Zahl, 1952; Nigrelli *et al.*, 1955; Chanley *et al.*, 1955) employing partially purified preparations that pointed to an antitumorous activity as well as a spectrum of other pharmacological actions. Among the latter, sufficient evidence of action against nerves was found to warrant the present study of the crystalline material on the propagated action potential in desheathed bullfrog sciatic nerve, on single nerve-fiber preparations from the frog, and on the rat nerve-diaphragm preparation.

Methods

The holothurin samples employed were prepared in the Mount Sinai Hospital laboratories, starting with the sun-dried Cuvierian glands of the sea cucumber. Using the fractionation techniques previously described (Nigrelli *et al.*, 1955), the crystalline material was obtained in analytical purity. It appears to be a steroid glycoside with four different sugar residues attached to the basic steroid nucleus. Other features of its structure include a half-esterified sulfuric acid moiety, and conjugate unsaturation in the aglycone. Solutions of holothurin were prepared freshly before use, and proved to be stable in storage at refrigerator temperatures. A molecular weight value of 1150 was used for the glycoside, corresponding to the observed acid equivalent weight.

Intravenous injection into mice. A highly inbred, NMRI strain of white mice was employed for the observation of toxic signs produced on injections of holothurin A via the tail vein. Male animals in the 18- to 22-gm. weight range were used exclusively, and injected doses on the weight basis (mg./kg. of body weight) were made with freshly prepared solutions of holothurin in physiological saline. Ten animals were injected at each dosage employed over the total 5 to 15 mg./kg. range producing mortality.

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Measurements with bullfrog sciatic nerve. Experiments designed to measure the relative activity of holothurin in effecting blockade of the propagated impulse in desheathed bullfrog sciatic nerve were carried out by techniques recorded previously (Moore *et al.*, 1958; Friess *et al.*, 1958). Observations were made at room temperature (24 to 25° C.) using two buffer systems (Ringer phosphate of *p*H 7.5 to 7.6 and Ringer bicarbonate at *p*H 8.1). Determinations with each buffer were scheduled to define the effect of *p*H on the strength of holothurin's blocking action. No correction for the effect of *p*H on the nerve was required since it has previously been noted that, within the *p*H 7–8 region, the nerve exhibits constancy of spike height and velocity.

In each experiment the extent of depression of the propagated impulse distal to the drug chamber was compared with the height of the control spike recorded proximal to the segment immersed in the drug, and the comparison expressed as percentage attenuation. Measurements were also made by the techniques of Moore *et al.* (1958) of the velocity of impulse conduction before, during, and after the action of a given drug, with the final observations of conduction velocity being made about an hour after washing of the preparation with fresh Ringer solution. For comparison, identical experiments using procaine, cocaine, purified veratridine, and physostigmine were performed, with the results also summarized in tabular form. These data serve to supplement the extensive studies of Skou (1954, 1956) on blockade of peripheral nerve with local anesthetics.

The technique employed in stimulation of the nerve preparation was essentially that described previously (Moore *et al.*, 1958). A voltage sufficient to cause firing of all A fibers was selected for a given nerve at the beginning of an experiment and kept constant throughout the entire experiment.

Phrenic nerve diaphragm. The conventional rat phrenic nerve-diaphragm preparation (Bülbring, 1946) in a chamber thermostatically regulated to 37° C. was employed. The bathing medium was a modified Ringer-Locke solution kept saturated with 95 per cent O_2 and 5 per cent CO_2, and buffered to *p*H 7.4 with bicarbonate. Stimuli were delivered as square waves of 0.1-msec. duration, alternately to the nerve and to the muscle from two synchronized Grass Stimulators. Maximal stimuli were used for each and applied at a rate of 1.5/min. to the muscle and the nerve. For comparison of species response the nerve-diaphragm preparation from the newborn kitten was employed. A diagram of the apparatus used is given in FIGURE 1.

Single fiber studies on frog nerve. In these studies the single fiber-single node preparation from one branch of the nerve controlling the semitendinosus-sartorius musculature was employed, as described by Tasaki (1953). In addition to observations with holothurin, a series of comparable experiments utilizing veratridine and physostigmine was included for purposes of contrast.

Results

Toxicity in mice. The results of intravenous administration of holothurin A to male white mice are summarized in TABLE 1 in terms of animal deaths per 10 test mice used at each dose level. It may be seen from the data of TABLE 1 that the dose-response curve has a rapidly rising slope near the LD_{50}

point of 9 mg./kg. dosage. The toxic signs develop with great rapidity, and culminate in death within 1 min. at dosages near the LD_{50} point. These signs include: thrashing convulsions, muscles taut, total body contortions, legs splayed and stiff, back humped, and finally, considerable respiratory difficulty.

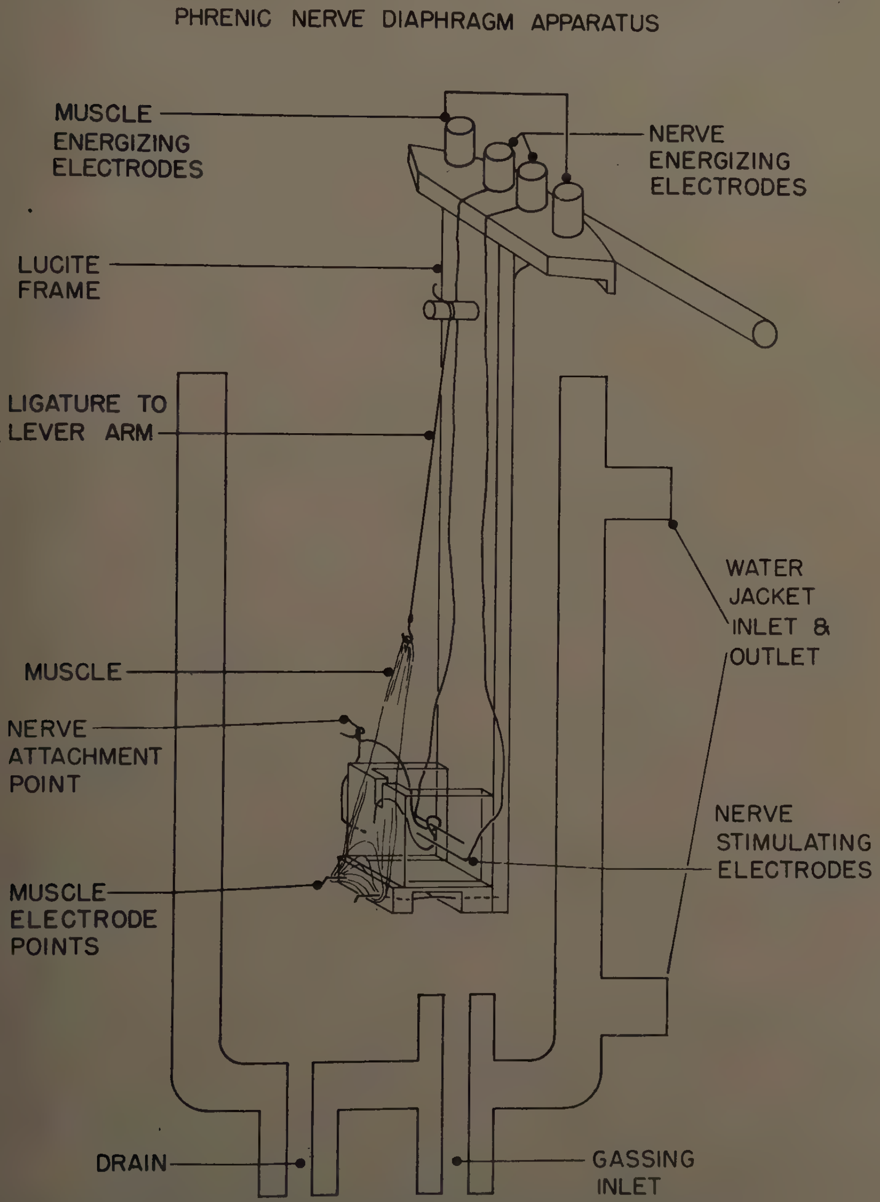
PHRENIC NERVE DIAPHRAGM APPARATUS
MUSCLE ENERGIZING ELECTRODES
NERVE ENERGIZING ELECTRODES
LUCITE FRAME
LIGATURE TO LEVER ARM
WATER JACKET INLET & OUTLET
MUSCLE
NERVE ATTACHMENT POINT
NERVE STIMULATING ELECTRODES
MUSCLE ELECTRODE POINTS
DRAIN
GASSING INLET

Figure 1. Diagram of the apparatus used in the phrenic nerve-diaphragm work. Apparatus designed by F. G. Standaert.

In all, the possible involvement of muscle and nerve elements in the total syndrome produced in this species sharpened the inherent interest in the later findings on isolated preparations from mammalian and amphibian species.

Desheathed bullfrog sciatic nerve. TABLE 2 summarizes the results of the study of the action of holothurin on the propagated action potential of this preparation and provides comparative data on the effects of cocaine, procaine, veratridine, and physostigmine. The table presents the percentage attenuation of the height of the propagated impulse by a given agent, the degree of recovery after washing the preparation with Ringer's solution of the appropriate *p*H, and the velocity of impulse propagation prior to treatment, at the time of maximum attenuation, and 1 hour after washing. It may be noted from the table that holothurin is quite effective in decreasing the magnitude of the propagated potential of this preparation in an action whose extent is concentration dependent and completely irreversible. Of particular note is the fact that even when the potential is markedly reduced in size, there is no reduction in the conduction velocity. In fact, the data for the action of holothurin in the

TABLE 1

HOLOTHURIN A TOXICITY IN MICE

I.V. dose, mg./kg.	Deaths/10 animals
12	9
10	7
8	4
7	2
6	0

TABLE 2

BLOCKADE OF DESHEATHED BULLFROG SCIATIC NERVE BY HOLOTHURIN AND OTHER AGENTS*

Agent	*p*H†	Drug concentration (mmole)	Percentage attenuation of spike height after 30 minutes (%)	Velocity of propagation‡ (cm./sec.)			Percentage recovery of spike height one hr. after washing
				Before drug addition	After drug addition	One hr. after washing	
Procaine	7.6	2.4	43	2.6	1.8(45)	2.4	100
Cocaine	7.5	1.2	42	2.8	2.0(45)	2.5	90
Veratridine	7.6	1×10^{-3}	74	3.0	2.9(20)	2.6	41
Physostigmine	7.6	8.0	46	3.4	2.5(23)	3.0	70
Holothurin	8.1	3.90	83	3.3	3.5(45)	3.4	0
	8.1	1.95	64	2.8	3.1(45)	3.3	0
	7.6	1.95	63	3.0	3.2(45)	3.2	0
	7.6	0.98	25	3.0	3.0(45)	3.1	0
	7.5	2.5	74	3.3	3.1(45)	3.6	0

* All observations made at 24 to 25° C. Values quoted are the results of individual experiments.

† Phosphate Ringer medium used for *p*H 7.5–7.6 experiments; bicarbonate Ringer used at *p*H 8.1.

‡ Estimated error in measurement: ±0.1.

Figures in parentheses represent minutes of contact time with blocking agent.

concentration range 1.95 to 3.90 mmole (TABLE 2) affords a bare suggestion that the conduction velocity may be slightly increased, but this increase is quite marginal in view of the estimated experimental error of measurement. To whatever extent this increment in velocity is real, it could be related to a phenomenon, discussed by Shanes (1958), in which narrowing of the wave form and consequently an increase in computed conduction velocity is the result of superposition of multiple impulses in the drugged, polyfiber preparation. It may also be noted from the table that while veratridine is somewhat similar, in that it too attenuates the potential without depressing the conduction velocity, cocaine, procaine, and physostigmine are distinctly different in this respect, and they markedly diminish conduction in doses that decrease the potential. In terms of potency it may be seen that veratridine is the most potent of this group, with holothurin about one thousandfold less active and approximately equivalent to cocaine, procaine, and physostigmine. The effect of *p*H on the action of holothurin is apparently very slight, since the change from *p*H 7.6 to *p*H 8.1 at a fixed concentration of 1.95 mmole produced no significant difference in effect.

TABLE 3
COMPARATIVE BLOCKING ACTIONS ON THE SINGLE FIBER-SINGLE NODE PREPARATION FROM FROG NERVE*

Drug	Concn. (mmole)	*p*H	Time (min.) required to reduce action current i to	
			$\frac{1}{2} i_0$	Zero
Veratridine	0.1	7.6	30	
	0.05	7.6	18–20	
	0.025	7.6	45	
Holothurin	1.0	7.3		2
	0.5	7.3		4
	0.1	7.6	7	10
	0.05	7.6	13	18
	0.025	7.6	>1 hr.†	
Physostigmine	10.0	6.7	i dropped 22% in 60 min.‡	

* Values quoted are the results of individual experiments.
† Action current reduced about 20 per cent.
‡ Recovered on washing.

Single fiber preparation with the frog nerve. The results of a series of experiments are summarized in TABLE 3, in which it may be seen that holothurin is moderately potent in diminishing the action current of the isolated node. The process is characterized by little or no lag after addition of the material, steady fall in the action current (the rate of which is concentration-dependent), and a concomitant rise in stimulation threshold. The effect on the single fiber, as on the nerve bundle, is irreversible. Indeed, once the decline in action current has begun, replacing the medium with fresh Ringer's solution devoid of holothurin does not halt the progression of the blockade. Veratridine is seen to be quite similar in action, but slightly less potent than holothurin. Its effect, too, is irreversible. Physostigmine, on the other hand, is considerably less potent and its effect is reversible upon washing. All of these agents are much less potent on this preparation than is cocaine, which has been reported by Tasaki (1953) to produce complete blockade at a concentration as low as 0.002 mmole.

An additional series of experiments with the single node preparation was designed to test whether the presence of a reversible blocking agent such as physostigmine could protect the node against the irreversible action of holothurin. To this end, a node was first brought to steady state blockade with 10 mmole physostigmine in the Ringer medium of *p*H 7.6, following which the bathing solution was replaced with Ringer's solution 10 mmole in physostigmine and 0.1 mmole in holothurin. Within 1 min. the action current fell to zero, accompanied by an equally precipitous rise in threshold voltage; repeated washing of the node with Ringer's solution proved unable to effect any recovery. It would appear therefore that a 100:1 ratio of physostigmine to holothurin is completely unable to block the irreversible effects of holothurin. Repetition of this experiment with 20 mmole physostigmine and 0.05 mmole holothurin (a ratio of 400:1) gave a similar result, namely a complete lack of protection against irreversibility of action on the part of the steroid derivative.

TABLE 4

EFFECT OF HOLOTHURIN ON NERVE-DIAPHRAGM PREPARATION*

Animal	Holothurin ($M \times 10^{-5}$)	Twitch height (mm.)				Percentage decrease in response		Permanent contracture (mm.)
		Control		After holothurin				
		N	M	N	M	N	M	
Rat	0.50	31.5	31.5	31	31	2	2	0**
Rat	1.0	42	21	28	14	33	33	7
Rat	2.0	23	18	12	9.5	48	47	11
Rat	2.0	28	28	21	22	25	21	8
Rat	2.5	†	33	21	22		33	8
Rat	4.0	27	30	18	9	33	35	8
Rat	4.0	29	29	19	23	39	21	6
Rat	6.0	25	26	0‡	4	100	85	12
Rat	10.0	28	16	1.5	5.5	95	66	9
Kitten	5.0	26	§	23	§	14		9

* N = nerve stimulation; M = muscle stimulation. Values quoted are the results of individual experiments.

** No change in height of base line, but downward overshoot decreased by about 6 mm.

† Stimulated muscle only at start.

‡ Nerve knocked out quickly, after holothurin addition.

§ Not possible to effect nerve and muscle stimulation separately in this preparation.

Phrenic nerve diaphragm. This phase of the study showed that holothurin is also very active in mammalian tissues, both nerve and muscle. A summary of the data obtained from the rat and kitten preparations is given in TABLE 4. A representative kymograph tracing showing the effects of holothurin at a concentration of 10.0×10^{-5} M is shown in FIGURE 2. Since the rat preparation was found to be considerably more sensitive to the material than that from the kitten, the bulk of the data and the discussion are based on this species.

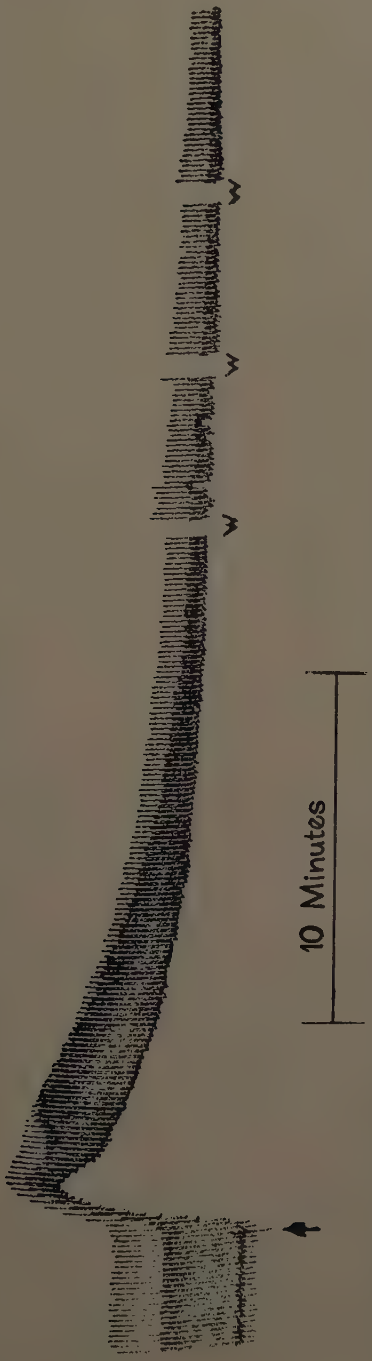

FIGURE 2. The action of 10.0×10^{-5} *M* holothurin on the rat phrenic nerve-diaphragm preparation. Breaks in the recording (W) indicate intervals in which the preparation was washed and the bath replaced with normal Ringer's solution containing no holothurin. Holothurin was added at the *arrow*. In each pair of twitches the nerve-stimulated twitch is recorded as the darker trace.

Several interesting features may be noted in FIGURE 2. First, the material promptly initiates a strong contracture which, although not shown here, was found to develop independently of the electrical stimuli; that is, a contracture of similar magnitude and time course was produced in unstimulated muscles and in strongly curarized preparations. Of note is the fact shown by the figure that the muscle, even at the peak of this contracture, is still capable of responding to electrical stimulation with a twitch. The contracture is not well maintained, but partially subsides in the course of 10 to 15 min. Interestingly, the magnitude of the residual contracture is independent of the holothurin concentration, as shown in the last column of TABLE 4.

With the decline in the strength of the spontaneous contracture there is a concomitant decline in twitch strength. This is a progressive process, the rate of which is initially comparatively rapid, but one that becomes very slow after about 30 min. (FIGURE 2). For purposes of comparison all the measurements in the table were taken at the 30-min. time point. It may be seen that the blockade of both the nerve and the muscle are roughly proportional to the initial concentration of holothurin (column 4, TABLE 4), with the effect on the nerve-elicited twitch somewhat greater than that on the directly-stimulated twitch. As in the amphibian preparations, this effect is irreversible. Repeated washings with fresh Ringer's solution not only fail to restore the full twitch, but do not stop the progression of the blockade or remove the contracture (FIGURE 2).

Discussion

The behavior of holothurin on these preparations presents some interesting parallels and contrasts. Since it is structurally a member of the saponin family it is not surprising to find that it displays pronounced activity against nerves. In related work with saponin, for example, Sato and Schneider (1954) observed that action currents recorded through the sheath of the isolated frog sciatic nerve were rapidly increased through the action of the steroids, with a concomitant decrease in conduction velocity and conspicuous changes in the gross appearance of the myelin sheath up to the point of conduction failure.

In the present work utilizing desheathed preparations a similar irreversible blockage of nerve function is found. Unlike the previous observations, however, no diminution of conduction speed is seen even at the time of marked attenuation of the height of the propagated impulse. The most likely reason for this difference in behavior is that while the other agents affect the function of all fibers in the polyfiber nerve bundle simultaneously, holothurin produces a complete and rapid blockade of some fibers but allows the remaining ones to function normally.

The mechanism of the blockade in these preparations is presently unknown. It may be a nonspecific surface action on the cell membrane, or it may be a specific attack at one or more receptor sites on the membrane. It should be noted, however, that unlike the action of saponin on the sheathed nerve preparation, holothurin does not cause any deterioration in the gross appearance of the individual nerve fiber even after prolonged contact.

Another interesting feature of the behavior pattern of holothurin is well illustrated in FIGURE 2. The glycoside appears to have a direct contractural effect on the muscle of the nerve-diaphragm preparation. This behavior recalls the effects of veratrine (for example, Kuffler, 1946) on the frog sartorius fiber, in which it was found that an initial electrical tetanus of short duration gives way to a sustained depolarization associated with contracture. With veratrine, electrical stimulation is required for the development of contracture, whereas holothurin produces its contracture independently of such stimulation.

Summary

The actions of the steroidal glycoside holothurin on amphibian nerve preparations and a mammalian nerve-muscle preparation have been studied. In general, the saponin has a powerful and irreversible action on both types of nerve, and appears to exert a direct, contractural effect on muscle.

Specifically, holothurin is comparable to the reference blocking agents cocaine, procaine, and physostigmine in potency on desheathed bullfrog sciatic nerve. However it affords a marked contrast with these reference agents in the sense that its action on this preparation is quite irreversible on washing, and it does not alter impulse conduction velocity in the course of its attenuation of the impulse. It displays the same irreversibility of action with respect to blockade of the action current in single fiber-single node nerve preparations from the frog.

On the rat phrenic nerve-diaphragm preparation, this material irreversibly blocks twitch response via either direct or indirect stimulation paths. Additionally, it displays a direct contractural effect on the muscle.

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FURTHER STUDIES ON THE STRUCTURE OF HOLOTHURIN*

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Some species of sea cucumbers (class Holothuroidea) contain a poisonous principle. The ecological significance of this poison is not clear. It has been suggested that it protects these animals against predators. Sea cucumbers of various species are eaten in some parts of the world, but occasional poisoning by this food is presumably not related to the toxin under discussion, which is ineffective by oral ingestion. A peculiar gland found in these species and named after Georges L. C. F. D. Cuvier is particularly rich in poison, which is also found in the brownish slimy integument. We have studied the chemical composition of the toxic substance from Cuvier's gland of *Actinopyga agassizi*, a holothuria found in the Bahama Islands. In two preliminary notes[1] we have reported that the toxic material holothurin, obtained from the water extract of Cuvier's gland, appears to consist of a few steroid aglycones, bound individually to four molecules of monosaccharides. Analysis of purified holothurin, designated holothurin A, and of its hydrolytic products, is in accord with this view.[2] This would place holothurin A into a class of cardiac glycosides or steroid saponins such as have hitherto been found only in plants, especially monocotyledons. It shares with the plant drugs its saponinlike character and neurotoxic and hemolytic properties, which are described elsewhere[3] and by Friess *et al.* in this monograph. In addition, holothurin contains one molecule of sulfuric acid, bound in ester linkage, which suggests a relationship with steroid alcohols, such as scymnol and ranol, in the bile of the most primitive vertebrates.

Holothurin A shows no absorption in the ultraviolet region. The infrared spectrum shows bands at 5.72 and 6.14 μ, indicative of a five- or six-membered ring lactone and one double bond. The absence of a positive Legal's test in conjunction with the ultraviolet spectrum excludes the presence of an α,β-unsaturated lactone. Hydrogenation of the double bond fails under a variety of conditions. Elementary analysis of holothurin A leads to the formula $C_{50\text{-}52}H_{81\text{-}85}O_{5\text{-}6}SNa$. Methoxyl determination indicates the presence of one such group in holothurin A. Acid hydrolysis of the neutral nonreducing holothurin A yields water-insoluble aglycones, sulfuric acid, and water-soluble reducing sugars. At least four individual steroid aglycones are obtainable from the hydrolysis of holothurin A. The elementary analysis and molecular weight determination of these aglycones show between 26 and 28

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carbon and 4 to 5 oxygen atoms, while none of the aglycones contains a methoxyl group. The observed rotation of the total sugar mixture, obtained from the acid hydrolysis of holothurin A, has been found to be the same as that calculated for an equimolecular mixture of the four sugars isolated and described below. These observations are consistent with the hypothesis that holothurin A is a mixture of several closely related sulfate ester glycosides, each of which contains a steroid aglycone of about 26 to 28 carbon and 4 to 5 oxygen atoms, one molecule each of four different sugars, and one molecule of sulfuric acid as the sodium salt.

The sugars obtained on acid hydrolysis of holothurin A were separated by chromatography on a cellulose column and identified by analysis, rotation, paper chromatography, melting point, and mixed melting points with authentic samples. They were xylose, glucose, 3-O-methylglucose, and glucomethylose (quinovose).

TABLE 1

ENZYMATIC HYDROLYSIS OF HOLOTHURIN A WITH *HELIX POMATIA*

Time (hours)	Hydrolysis (%)	
	Run 1	Run 2*
0	—	—
3	3	—
23	13	23
45	22	35
64†	25	37
143	36	37
170†	37	—
189	39	—

Holothurin A (100 mg.) + $H^+ \rightarrow$ 51 mg. of sugar mixture.
* Twice as much enzyme used as in Run 1.
† Additional enzyme added.

In order to determine the sequence of these four sugars in holothurin A, we subjected it to enzymatic hydrolysis at *p*H 5.2 with an extract of *Helix pomatia.** In TABLE 1 a summary of the course of the enzymatic scission is given. The percentage of enzymatic hydrolysis is calculated from the reducing values observed with Nelson's reagent and that found after total hydrolysis with acid. Approximately one third of the reducing value found in the acid hydrolysate is due to xylose. Since very little xylose and di- and trisaccharides of intrinsically lower reducing capacity are found in the enzymatic hydrolysate, it may be said that at least about 75 per cent of the monoses, glucose, 3-O-methylglucose, and quinovose have been removed from holothurin A. The insoluble compounds produced by the enzymatic scission are now under investigation. One of them appears to contain only xylose and sulfuric acid attached to the aglycone.

The sugar components of the enzymatic hydrolysate were characterized by

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* The enzyme mixture "glusulase" was obtained from Endo Products, Inc., Richmond Hill 18, N. Y. In addition to glycosidases, it contains β-glucuronidase and sulfatase.

TABLE 2

CHROMATOGRAPHIC SEPARATION AND CHARACTERIZATION OF SUGAR MIXTURES

R_f in phenol/H_2O	Sugar	Sugar (R_f in Bu/Et/H_2O)	Final products
0.39	Glucose plus 3-OMe-Glucosyl-glucosyl-xylose (trace)	— Same (0.14) $\xrightarrow{H^+}$	Glucose plus xylose (trace)
0.59	Quinovose	— Quinovose (0.34) $\xrightarrow{H^+}$	Quinovose
0.72	3-OMe-glucose plus 3-OMe-glucosyl-glucose plus glucosyl-xylose (trace)	3-OMe-glucose (0.34) $\xrightarrow{H^+}$ 3-OMe-glucosyl-glucose (0.15) + glucosyl-xylose (trace) $\xrightarrow{H^+}$	3-OMe-glucose Glucose plus 3-OMe-glucose plus xylose (trace)
0.83	Quinovosyl 3-OMe-glucose	— Same (0.40) $\xrightarrow{H^+}$	Quinovose plus 3-OMe-glucose

TABLE 3

CHROMATOGRAPHIC SEPARATION AND CHARACTERIZATION OF SUGAR MIXTURES

R_f in Bu/Et/H_2O	Sugar	Sugar (R_f in phenol/H_2O)	Final products
0.15	Glucose plus 3-OMe-glucosyl-glucose plus glucosyl-xylose (trace) plus 3-OMe-glucosyl-glucosyl-xylose (trace)	Glucose plus trisaccharide (0.40) 3-OMe-glucosyl-glucose plus glucosyl-xylose (0.70)	Glucose plus xylose (trace) Glucose plus 3-OMe-glucose plus xylose (trace)
0.34	Quinovose plus 3-OMe-glucose		
0.40	Quinovosyl-3-OMe-glucose	Same (0.82)	

TABLE 4

SUGARS FROM ENZYMATIC HYDROLYSIS

Monosaccharides	Disaccharides	Trisaccharides
Xylose (absent)	Glucosyl-xylose (trace)	3-OMe-Glucosyl-glucosyl-xylose (trace)
Glucose	3-OMe-glucosyl-glucose	Quinovosyl-3-OMe-glucosyl-glucose (not isolated)
3-OMe-glucose	Quinovosyl-3-OMe-glucose	
Quinovose		

paper chromatography using two solvent systems, phenol/H_2O and butanol/ethanol/H_2O. Chromatography of the sugar mixture on Whatman No. 1 paper, employing the phenol/H_2O system, gave four distinct spots after development of color with aniline phthalate. The R_f values were 0.39, 0.59, 0.72, and 0.83. Chromatography of the sugar mixture, employing butanol/ethanol/H_2O, gave three distinct spots of R_f values 0.15, 0.34, and 0.40. Xylose has an R_f of 0.45 in phenol/H_2O and 0.25 in butanol/ethanol/H_2O, and gives a distinct red coloration with aniline phthalate. The presence of free xylose was thus excluded. Each of these spots was eluted from the paper and rechromatographed with the alternate system; furthermore, each eluate was hydrolyzed with acid and the hydrolysate then rechromatographed using both systems. The results of these chromatographics are summarized in TABLES 2 and 3. Glucose, quinovose, and 3-O-methylglucose are found in the free state. The disaccharides quinovosyl-3-O-methylglucose and 3-O-methyl-glucosyl glucose have been positively identified. Since xylose appears only after acid treatment of the enzymatic hydrolysates in trace quantities, as indicated in the tables, we have assumed that both a disaccharide and a trisaccharide containing xylose are present in the mixture. The positive identification of the disaccharides, quinovosyl-3-O-methylglucose and 3-O-methyl-glucosyl-glucose, then allows of only one sequence in holothurin A: quinovose, 3-O-methylglucose, glucose, xylose, and steroid (TABLE 4).

Only trace quantities of sulfate were found in the enzymatic hydrolysate. We conclude from this that the sulfate moiety is linked to the aglycone portion of holothurin A.

Summary

In order to determine the sequence of the four monoses in holothurin, we subjected it to enzymatic hydrolysis with an extract of *Helix pomatia*. The following fragments were isolated from the hydrolysate: quinovose, 3-O-methylglucose, traces of glucose, quinovosyl-3-O-methylglucose, 3-O-methyl-glucosyl-glucose and small amounts of 3-O-methyl-glucosyl-glucosyl-xylose. Hence the sequence of the monoses is quinovose, 3-O-methylglucose, glucose, and xylose, the last named being attached to the aglycone. Little free sulfate was found in the enzymatic hydrolysate, indicating that the sulfuric acid is not attached to the sugar moiety, but to the steroid aglycone.

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STEROLS OF SOME INVERTEBRATES*

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The early investigators of sterols were plagued by a lack of analytical tools and methods for isolating sterols found in the plants or animals they investigated. The usual method of isolating sterols was to crystallize them from alcoholic solutions of the nonsaponifiable material of the species under investigation. If there was only one major sterol, this procedure was, of course, satisfactory, but many of the sterols reported in early literature were actually mixtures of sterols.

Almost sixty years ago Henze[1] isolated a sterol from the Mediterranean sponge *Suberites domuncula*, the first sterol from an animal that was clearly shown to be different from cholesterol. Appropriately, this sterol was named spongosterol. Five years later, in 1909, Dorée[2] reported the isolation of another new sterol, clionasterol, from the sponge *Cliona celata*, obtained from the nonsaponifiable matter by crystallization from methanol. The investigation of sterols in sponges stopped until 1933, when Bergmann[3] reported the finding of a new sterol from the red New England sponge *Microciona prolifera*, which he named microcionasterol. The sterol was isolated by precipitating it from an alcoholic solution of the nonsaponifiable matter.

When the spongosterol of Henze was reinvestigated by Bergmann *et al.*,[4] it was found that there were actually two major sterols. One was a saturated sterol, and the other was a monounsaturated sterol. The saturated sterol was found to be cholestanol, and the unsaturated sterol was named neospongosterol. Neospongosterol has a saturated ring system, and a double bond in the side chain in the C_{22} position. It was the first 28-carbon sterol to be isolated from sponges, and the first unsaturated one to be isolated with a saturated nucleus. Also, this was the first time that cholestanol had been found to be a major sterol in any representative of the animal world. The structural formulas for both sterols are shown in FIGURE 1. The sponge that Henze worked on was *S. domuncula*, while the one studied by the Bergmann group was *S. compacta*, but they are closely related, and the spongosterol isolated in each case was similar in every respect.

The Bergmann group isolated clionasterol from the loggerhead sponge[5] *Spheciospongia vesparis*, which proved to be identical to the clionasterol isolated by Dorée from *C. celata*. Further investigation on either sample of clionasterol proved that it was a mixture containing a small quantity (less than 1 per cent) of a sterol with conjugated double bonds, and two major sterols, one mono- and one diunsaturated sterol. These sterols were separated by way of their acetate bromides, according to the method of Windaus.[6]

The diunsaturated sterol, which amounted to about 40 per cent of the total

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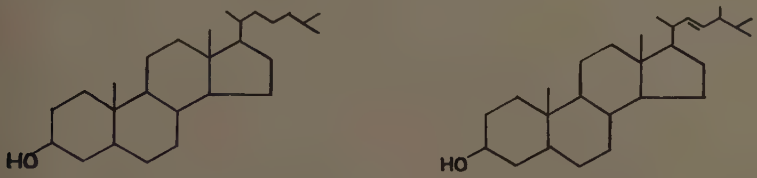
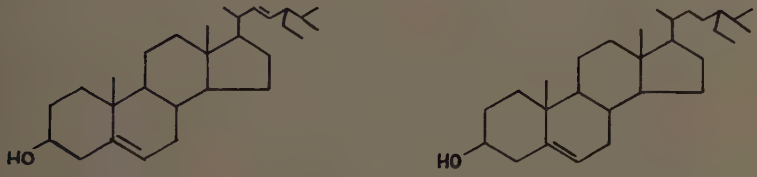
† Deceased, November 2, 1959.

sterol mixture, was named poriferasterol, since it is a typical representative of sterols in the phylum Porifera. The formula of the sterol $C_{29}H_{48}O$ was determined by careful analyses of a series of derivatives. The structural formula is shown in FIGURE 1.

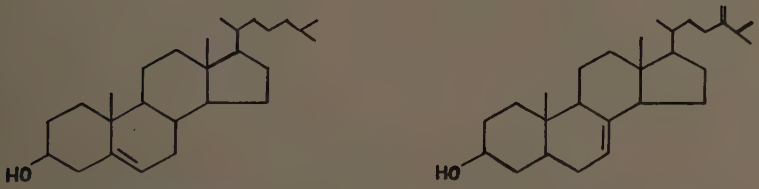
The monounsaturated sterol, which amounted to about 60 per cent of the total sterol mixture, retained the name clionasterol and, on the basis of the analysis of derivatives of the sterol, the formula $C_{29}H_{50}O$ was assigned to the sterol. The structural formula is shown in FIGURE 1.



Cholestanol **Neospongosterol**



Poriferasterol **Clionasterol**



24-Methylene cholesterol **7,24(28)-Ergostadien-3β-ol**

FIGURE 1. Some sterols of invertebrates.

When the Bergmann group reinvestigated the sterol of *M. prolifera*,[7] they found that microcionasterol was a mixture of 35 per cent cholesterol, 40 per cent cholestanol, a diunsaturated sterol, and a second monounsaturated sterol, neither of which was identified.

The use of chromatographic methods greatly simplified the problems involved in isolating sterols. In 1933 Winterstein and Stein,[8] in a discussion of chromatographic separations, mentioned that separations of sterols could be effected on alumina columns if there were differences in the number of double bonds. In 1938, Wallis[9] and his co-workers described the preparation of colored *p*-phenylazobenzoyl (azoyl) esters of cholesterol, β-sitosterol, ergosterol, and stigmasterol, and the separation of mixtures of the esters on alumina columns. The separation of a mixture of the esters of cholesterol and β-sitosterol was not possible, since the total number of double bonds in the molecule appeared to be the important factor in the separation. The only difference in the two molecules is the ethyl group in the C_{24} position on β-sitosterol, in place of a hydrogen in the cholesterol molecule. A mixture of 1 part of benzene to 10 parts of high boiling petroleum ether was used as the solvent. The advantages of this method are that with these colored compounds one can watch the separation visually, and the sterols can be readily regenerated by a simple saponification of the esters.

Idler and Baumann[10] modified the Wallis method, and used silicic acid and celite as the adsorbent to separate azoyl esters. Using this method, Fagerlund and Idler[11,12] have isolated several new sterols. For example, 24-methylene cholesterol has been isolated from the clam *Saxidonus giganteus*, from the oyster *Ostrea gigas*, and from several other mollusks. When 24-dehydrocholesterol was isolated from the barnacle *Balanus glandula*,[13] this was the first sterol other than cholesterol to be isolated from a crustacean. The starfish *Pisaster ochraceus*[14] yielded another new sterol, 7,24(28)-ergostadien-3β-ol. The structural formulas of these sterols are shown in FIGURE 1.

In our laboratory, a new method for separating sterols has been developed. It was observed that *p*-phenylazophenyl isocyanate, prepared according to the method described by Raiford and Freyermuth,[15] reacts quantitatively within fifteen minutes in boiling hexane with sterols to give colored urethanes that can be separated by chromatography on florisil columns, using 30 per cent benzene and 70 per cent heptane as the solvent. The urethanes of cholesterol, ergosterol, and stigmasterol were prepared as model compounds; mixtures of any two of these urethanes and mixtures containing all three urethanes can be easily separated. The urethanes can be cleaved with $LiAlH_4$ in tetrahydrofuran to quantitatively give back the free sterol. The method is superior to previously reported methods, since the urethanes are colored, and all of the reactions are quantitative.

Using this new method, a new sterol has been isolated from the slate pencil sea urchin. The sterol was also isolated by chromatography of the azoyl esters of the nonsaponifiable matter. The melting point of the sterol is 121.8 to 123.0° C., the optical rotation ($[\alpha]_D$) is $-10.5°$, the melting point of the acetate derivative is 115 to 116° C., and the Liebermann-Burchard test indicates that the sterol is a "fast-acting" or Δ^7 sterol. The infrared spectrum shows no unsaturation in the side chain. When bromine in glacial acetic acid was added to a portion of the sterol in an ether solution, no precipitate formed. Due to the limited amount of material available, no further chemical work could be done.

The survey of the distribution and structure of animal sterols remains far from complete. Many phyla have not been investigated at all, and the available information on many other phyla is still very inadequate. What is the origin of these sterols? It is probable that invertebrates dependent upon exogenous sterols convert them to the compounds characteristic of their classes and families regardless of their feeding habits.

The most primitive animals, such as the sponges, contain the most diverse collection of sterols. Cholesterol is included in this diverse collection, but it is neither the most typical nor the most prominent sterol of lower invertebrates. Higher on the evolutionary ladder other sterols disappear, and cholesterol becomes more prominent until, in the vertebrates, it is the only principal sterol. Bergmann[16] has suggested that the reduction of the multitude of closely related compounds performing closely related functions to a few compounds, or to one compound, is a general phenomenon of biochemical evolution. Cholesterol may be viewed as representing the "survival of the fittest" sterol.

Why do most plant sterols and many sterols of invertebrates have substituents in the C_{24} position? Why do Δ^7 sterols predominate in some orders of lower animals? A better understanding of biosynthesis and metabolism of sterols, as well as a more detailed study of sterols, is needed to solve these problems.

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THE NUCLEIC ACIDS OF SPONGES*

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In recent years, Bergmann and his co-workers have examined extensively the lipid fractions of a large number of marine invertebrates with the view of elaborating a scheme of biochemical evolution. In 1950, while investigating the lipid fraction of the Caribbean sponge, *Cryptotethia crypta*, Bergmann and Feeney[3,4] found that a highly crystalline material separated from the initial acetone extracts of the dried sponge. Further investigation of this material showed it to be a mixture of several nucleosides. One of these, spongothymidine, was readily purified and shown to be a pentofuranoside of thymine. Because of its high dextrorotation, however, it was thought not to be the ribofuranoside of thymine, which was as yet unknown.

By means of chromatography, this mixture of nucleosides was finally separated, and shown to have three components: spongothymidine; spongouridine, a pentofuranoside of uracil; and spongosine, a riboside of an unidentified purine. Bergmann and Burke[1] were able to show, by degradative methods, that both spongothymidine and spongouridine were the β-D-arabofuranosides of thymine and uracil respectively. These findings have since been corroborated by synthesis in other laboratories.

Spongosine, the purine riboside, however, was more difficult to identify. It was easily shown to be a ribofuranoside by means of titration with sodium metaperiodate. The ultraviolet spectra of both the nucleoside and base showed two maxima in acidic solution, but only one in basic solution. This behavior had not been noted in any other known purine. An analysis of a sample of the purine showed, on the other hand, one more carbon atom than was expected for an unsubstituted purine. By comparing the ultraviolet spectra of the purine with those of other known purines and by eliminating other possible structures, it became evident that the only structure that would fit all the data was that of an O-methyl purine. At the same time, the action of adenosine deaminase on the nucleoside showed the presence of an amino group on position 6 of the purine ring system. Synthesis showed, indeed, that this purine had the structure 2-methoxyadenine.[2] The glycosidic linkage in the nucleoside was shown to be β by synthesis of the compound using orotonoside, isoguanosine, as starting material. This latter compound has a β-glycosidic linkage, which indicates that spongosine is the β-D-ribofuranoside of 2-methoxyadenine.

The presence of these three new nucleosides in the extracts of *Cryptotethia crypta* is very interesting. Were these compounds degradation products of some sort of nucleic acid, or were they parts of some as yet undescribed coenzyme? With the former question in mind, Bergmann *et al.*[5] undertook the investigation of a number of sponges, including *Cryptotethia crypta.*

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It might be mentioned here that up to 1957 only about 10 species of invertebrates had been examined with respect to their nucleic acids, and most of these were sea urchins. In all of these cases, the only difference noted in the composition of the nucleic acid was the partial replacement of cytosine by 5-methylcytosine in the DNA of several animals.

The nucleic acid fractions of about 16 species representing eight orders of sponges originating in Long Island Sound, the Bermuda Islands, and the Bahama Islands were investigated. These fractions were isolated by a number of conventional methods, and then subjected to degradation. In all cases, however, the base and carbohydrate composition of the nucleic acids were as usually found in ribo- and deoxyribonucleic acid. No traces of the *Cryptotethia* nucleosides nor of any arabinosides could be found in the nucleic acid fractions of any of these sponges. The purine:pyrimidine ratio of several of the sponge deoxyribonucleic acids was also determined. In all cases, including that of *Cryptotethia crypta* deoxyribonucleic acid, this ratio was found to be approximately 1:1, which is in accord with the requirements of the recently postulated Watson-Crick double helix structure for deoxyribonucleic acid.[6a,b]

It is important to note here that all the sponge nucleic acids that were isolated had a polysaccharide closely associated with them. This material was not easily separated from the nucleic acid mixture without substantial loss of nucleic acid. As this material did not interfere with the experimental results, no efforts were made to remove it. In one case, however, that of the sponge *Microciona prolifera*, acid hydrolysis of a partially purified sample showed the material to be substantially a polyglucoside, although this may not be true for the other sponges.

On the other hand, *Cryptotethia crypta* did show some very strange results in this investigation. This sponge was found to contain only very small quantities of ribonucleic acid, less than 4 per cent of the total nucleic acid isolated. The remainder was deoxyribonucleic acid. All the other sponges gave much more ribonucleic acid than deoxyribonucleic acid, which is the usual quantitative relationship between the amounts of these two materials. This is extremely interesting, as the amount of ribonucleic acid is small enough to be due to an extraneous source rather than the sponge itself. If this should prove to be the case, we have here an example of an animal with a very strange form of metabolism. It must be noted here that the sponges under investigation were stored at −30° C. before they were extracted, and in several cases periods of time of up to two years elapsed before the extractions were done. The *Cryptotethia crypta*, however, was the only sponge that showed this strange behavior, making it rather difficult to assume that the low yield of ribonucleic acid was due to some sort of slow enzymatic degradation. One would expect a similar sort of action in all of the sponges, not only one particular species.

A cursory investigation by means of chromatography of aqueous extracts of *Cryptotethia crypta* did not show the presence of any of the previously-described nucleosides in the form of phosphate esters, as might have been expected. This work, however, must be repeated with more care on freshly collected material before any definite conclusions may be drawn, but it would be interesting at this point to speculate that *Cryptotethia crypta* possesses no ribonucleic acid.

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ANTIMICROBIAL SUBSTANCES FROM SPONGES

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A marine biologist may look upon the phenomenon of antibiosis, with its broadest implications, as one involved in the limitation or exclusion of certain forms of life by diffusible substances produced by other living species. In the vast capacity of the ocean such materials set up activity gradients, sometimes against each other, and regulate by their presence the entire chain of dependent events (Nigrelli, 1958).

This antibiosis may extend its effects to the higher forms, such as many-celled invertebrates and fish, as in the case of some toxic substances, for example, holothurin, elaborated by the sea cucumber (Nigrelli, 1952). Antibiosis becomes of particular interest when materials exhibiting antimicrobial activity originate in the bodies of organisms adapted to live as filter feeders and to act as strainers to myriads of bacteria and microplankton.

Sponges are a classical example of such forms of life. Long regarded as plantlike in nature, they finally attained their status as an animal phylum in 1835, but retained a rather unique position that sets them apart from other multicellular animals because of their structural and developmental peculiarities. With the exception of one family, sponges are marine, and their natural food consists chiefly of minute organisms and organic detritus. There is also some speculation that they may utilize dissolved nutrients. In the absence of a digestive cavity, digestion is intracellular, and it takes place in the specialized collar cells, the choanocytes, and the amebocytes. Some of the products of digestion are stored as glycogen, fats, glycoproteins, and lipoproteins in amebocytes, and are then known as thesocytes. The excretory products are usually complex nitrogen bases, such as agmatine, a guanidine derivative. In view of their sessile habit and porous structure, sponges are frequently inhabited by a large variety of animals, including fishes, echinoderms, crustaceans, annelids, molluscs, bryozoans, coelenterates, and by other sponges. The interrelationships of these organisms are not known, but many of the associations are no doubt accidental, although commensalism, mutualism, and inquilinism have been established in a few instances (Hyman, 1940). Blue-green algae and cryptomonads in sponges have also been described (Lami, 1941, 1942).

In so far as is known, sponges are not generally used as food by fishes and only rarely by other animals, such as nudibranchs (Abeloos and Abeloos, 1932). The reason for this has not been determined, but it may be related to the fact that many sponges contain noxious substances. Sponges are also apparently resistant to the action of the multitude of microorganisms that enter their complex system of canals and cavities (spongocoels) and become part of the diet. Sponges, however, are highly sensitive to the reduction of available oxygen; under such conditions the animals change color and die within a relatively short time, decomposition setting in almost immediately. Gas exchanges have been studied by Hyman (1916, 1925), Houget *et al.* (1927), and von Ledebur (1939).

Starting in 1952, Nigrelli observed the antibiotic effects of some subtropical sponges by placing fresh sponge fragments on seeded Petri dishes. It was found later that fluids extracted from freshly collected sponges showed a relatively high degree of antibacterial activity against forms isolated from the spongocoel or the surrounding sea water, as well as against other organisms employed routinely for assaying such activity. Material extracted from dying or dead sponges showed little or no such activity, and the number of bacteria was seen to increase considerably within a relatively short time.

The naturally occurring bacterial flora of subtropical sponges was studied by placing fragments of fresh sponge with sea water on a nutrient agar medium, fortified with the extract of the same sponge. In this manner pure cultures were obtained of what appeared to be a single bacterial type from all except one species of sponge, which yielded two distinct types. It was assumed that the bacteria isolated with the aid of homologous sponge extracts were resistant to the specific antibiotic and hence were capable of surviving within a given sponge. Pure cultures of these bacteria were tested for activity against *Staphylococcus aureus* by streaking them across the plate containing the cocci, but no growth inhibition was observed. It was concluded that these bacteria represented forms adapted to the presence of the antibiotic but were not the source of the active substances.

These and other observations led us to believe that the sponges per se produce the materials possessing antimicrobial properties. One of these substances, extracted from the North Atlantic red-beard sponge (*Microciona prolifera*), has been previously isolated and named ectyonin (Nigrelli *et al.*, 1959).

Substances with varying degrees of antimicrobial activity were also obtained from two other temperate zone sponges, *Halichondria panicea* (crumb-of-bread sponge) and *Cliona celata* (gamma or free-living form of the sulfur sponge), and from the following subtropical species: *Tedania ignis* (fire sponge); *Haliclona viridis* (green sponge); *Dysidea etheria* (heavenly sponge); and *Oligocera hemorrhages* (bleeding sponge).

The active materials may be readily extracted from water suspensions that microscopically appear to consist of almost homogeneous cell populations. Most of these substances are heat-stable and can be extracted selectively from frozen and heat-dried sponges with a variety of organic solvents. Some are further separated into a number of components by paper chromatography and at present the purification and some chemical characterization of these materials is in progress. The activity of these materials ranges from general inhibition of a variety of naturally occurring sea water bacteria to highly specific action. The materials from all the sponges studied appear active against the Gram-negative *Escherichia coli.* Remarkable activity against poikilotherm and bovine forms of tubercle bacilli was exhibited by the extracts of the red-beard sponge (Ectyonin), by the crumb-of-bread sponge, fire sponge, bleeding sponge, heavenly sponge and, to a somewhat lesser extent, by the sulfur sponge. Activity against *Pseudomonas pyocyanea* was observed with extracts of the red-beard sponge and the bleeding sponge. *Candida albicans*, an imperfect fungus, was inhibited with ectyonin and with the extracts of the sulfur and fire sponge.

Among the sponges studied, the red-beard sponge is remarkable for its broad-spectrum antibiosis. Similar action was exhibited by the aqueous extracts of the green sponge, which were found antibiotic against all the microorganisms tested. However, in addition, the green sponge extract was found lethal to a variety of invertebrates, fish, amphibia, and mice in dilutions from 10 to 100 ppm, with no comparable toxicity exhibited by any of the sponges studied to date. The solution of the green sponge that caused death of the test fish within 1 hour was able to produce a 16-mm. zone of inhibition (measured from the margin of the disc) on *Staphylococcus aureus*, and the solution that caused death in 1.5 hours caused an inhibition zone of 14.5 mm.; hence the toxicity of the extracts to fish appeared related to the antibiotic activity *in vitro*. The material from the green sponge may eventually prove of interest. It consists of a number of fractions, and it may be hoped that one will be obtained that is not toxic to animals while retaining its antibiotic properties. Strangely, this highly toxic sponge under natural conditions harbors a tube-worm, *Spirorbis* sp., which lives within a self-built calcareous tube that may protect the worm from the harmful substance.

Of possible ecological interest are the interactions of the sponge extracts *in vitro* in respect to their joint ability to inhibit growth of *Staphylococcus aureus* as the assay organism. These relationships were tested in view of the possible interaction of this type between the materials elaborated by the sponges known to live in close ecological association. It has been found, for example, that dense colonies of the crumb-of-bread sponge are often intimately associated with *Microciona prolifera*. When the extract of the red-beard sponge, known to produce approximately a 1.0-mm. zone of inhibition, was combined with the water extract of the crumb-of-bread sponge, which by itself produced a 1.5-mm. zone of inhibition, the antibiotic effect was almost eliminated. Similarly, the bleeding sponge and the heavenly sponge are found living in the same habitat. The capacity of the bleeding sponge extract to produce a 2.0-mm. zone of inhibition disappears when the extract is combined with a slightly less active extract of the heavenly sponge. When the extracts of these two pairs of sponges are not combined on the same disc, but placed on separate discs at some distance from each other on the seeded plate, the two substances in each case appear to set up activity gradients; these become independent of each other when the distance between them reaches approximately 3.0 cm. When the active extracts from the fire sponge and the bleeding sponge, which also live in the same habitat, are combined, the antibiotic activity persists, but is not additive.

Of similar interest in respect to ecology is the *in vitro* interaction between the highly active (and toxic) extracts of the green sponge, and of the equally toxic holothurin, which per se does not exhibit antibiotic properties. When the substances produced by these close neighbors, the green sponge and the sea cucumber, are mixed in aqueous solutions at room temperature, the antibiotic activity of the sponge extract is suppressed. In the fish assay, on the other hand, when holothurin solution that produces death in 30 min. is combined with the green sponge solution that produces death in 156 min., death results in 18 min.

Although no detailed surveys have been made for each pair of such "antagonists," that is, sponges producing substances that inactivate or inhibit each other *in vitro*, the phenomena observed may be related to ecological succession. In support of this we may say that in the summer of 1959 *Halichondria panicea* became the dominant species, whereas *Microciona prolifera* was the most abundant in the previous year.

In view of the work in progress it appears that each one of the antibiotic materials obtained from these species of sponges actually consists of a number of separable factors; some of them do not exhibit antibiotic activity while others appear as specific growth inhibitors or growth stimulants. It is believed that in nature it is the balance of these factors that produces growth regulatory effects. The relative yields of the active materials from each species of sponge must be considered not in terms of total dry weight of the animals, but in terms of the total organic matter, characteristic for each species. It has been shown (Bergmann, 1949) that the heavenly and the bleeding sponges consist of 100 per cent organic matter, whereas *Microciona prolifera* yields only 55 per cent per total dry body weight. In this sense, the *in vitro* antibiotic action probably results from relatively low amounts of the active materials. Detailed studies on the *in vitro* action of the individual fractions from each sponge may give some clues as to the mechanism of antibiosis under natural conditions.

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CHEMISTRY OF COELENTERATES. I. OCCURRENCE OF TERPENOID COMPOUNDS IN GORGONIANS*

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As part of a study of the comparative biochemistry of marine invertebrates, we have examined a number of gorgonians (class Anthozoa; subclass Alcyonaria; order Gorgonacea) for the occurrence of nonlipid organic compounds. From certain species we have obtained crystalline as well as liquid substances that seem to be terpenoid compounds. These substances appear to account for the antibiotic activity of gorgonian extracts,[1] for part of the odor of the animals, and for the irritation of skin and eyes caused by handling gorgonians. Yields of the crystalline compounds ranged from 0.005 to 1.5 per cent of the dry weight of gorgonian cortex. The infrared spectra of the solid compounds show strong absorption in the carbonyl region, and the compounds appear to be lactones. The nonlipid oils obtained from the animals are unsaturated hydrocarbons with an admixture of ketonic material.

Methods and Results

The gorgonians studied include *Plexaura crassa* Ellis and Solander, *Pterogorgia americana* (Gmelin), and *Rhipidogorgia flabellum* (L.), obtained at Bermuda; *Briareum asbestinum* (Pallas), *Eunicea mammosa* Lamouroux, and *Xiphigorgia anceps* (Pallas) from Bimini; and *Leptogorgia setacea* (Pallas) from the Gulf of Mexico near Port Aransas, Texas.

The animals were dried as soon as possible after collection by hanging them in the shade on a line during the sunny part of the day and then keeping them in an oven at around 60° C. Drying was completed within 24 hours. The cortex was stripped from the animals and crushed to a coarse powder by using a meat grinder or a mechanical blender. The crushed cortex was extracted in large Soxhlet type extractors using *n*-pentane or ether. Crystalline material precipitated from the solutions during extraction, and was purified by chromatography or recrystallization as indicated below. The terpene hydrocarbons were isolated from the concentrated extracts by distillation *in vacuo* or by chromatography.

Melting points are uncorrected.

Plexaura crassa. P. crassa yields a considerable amount of material that crystallizes out during the extraction with pentane or ether. Recrystallization from ether and from carbon tetrachloride-hexane yields an optically-active, unsaturated, polycyclic compound, m.p. 144 to 145.5° C., of complex structure, which we have tentatively named crassin acetate, $[\alpha]_D^{25}$ $+70.7°$ (c = 4.0, ethanol).

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The pentane-soluble extract of *P. crassa* yields upon distillation *in vacuo*, followed by chromatography over alkaline alumina, a material boiling at 71° C. at 2.3 mm., having characteristic absorptions at 6.07, 11.46 and 12.15 μ, $[\alpha]_D^{31}$ $+24.5°$ (c = 6.0, $CHCl_3$), and 88.52 per cent C, 11.87 per cent H ($C_{15}H_{24}$ requires 88.16 per cent C and 11.84 per cent H). Determination of unsaturation, using perbenzoic acid titration, indicated the presence of two oxidizable bonds. When the hydrocarbon was treated with hydrogen chloride in ether (+)-cadinene dihydrochloride was obtained, m.p. 117 to 118° C., $[\alpha]_D^{31}$ $+36°$ (c = 0.75, chloroform). Its infrared absorption spectrum was identical with that of authentic (−)-cadinene dihydrochloride obtained from oil of cade. Analysis showed 64.85 per cent carbon, 9.47 per cent hydrogen, and 25.75 per cent chlorine; $C_{15}H_{26}Cl_2$ requires 64.97 per cent, 9.45 per cent, and 25.58 per cent, respectively. Cadalene (1,6-dimethyl-4-isopropylnaphthalene) was obtained in 15 per cent yield on dehydrogenation of the cadinene from *P. crassa* with selenium at 240° C. Identity was established by conversion to the picrate, m.p. 114 to 115° C., and to the trinitrobenzene adduct, m.p. 110.5 to 111° C.

Crassin acetate was cidal *in vitro* against *Endamoeba histolytica* at $^{1}/_{50,000}$. The volatile oil from *P. crassa* inhibited growth of *Clostridium feseri* and *Staphylococcus aureus.*

Eunicea mammosa. The insoluble material precipitating from pentane or ether extracts yielded a colorless crystalline solid in quantities approximating 1.5 per cent of the dry cortex. This compound, which we have tentatively named *eunicin*, melts at 150 to 152° C., is optically active, $[\alpha]_D^{29}$ $-95°$ (c = 2.0, ethanol), and shows infrared absorption maxima (chloroform) at 2.75, 5.65, and 5.99 μ, which indicate that the substance is an unsaturated, hydroxylated γ-lactone. Its empirical formula $C_{15}H_{22}O_3$ (analysis, calculated: C, 71.97 per cent; H, 8.86 per cent; found: C, 71.87 per cent; H, 8.90 per cent), and other properties, indicate that eunicin may be a sesquiterpene lactone.

In tests for bacterial activity, eunicin and the volatile oil from *E. mammosa* have been found to inhibit growth of *Staphylococcus aureus* and *Clostridium feseri.* Chromatography of pentane extracts of *E. mammosa* on Magnesol, followed by distillation *in vacuo*, has yielded an optically active unsaturated hydrocarbon mixture with an odor characteristic of *E. mammosa.*

Xiphigorgia anceps. Pentane extracts of *X. anceps* deposit a solid that appears to be a mixture of lactones, and which can be separated by chromatography in chloroform over alumina. The major component, $[\alpha]_D^{28}$ $+13.2°$ (c = 2.8, chloroform), m.p. 93 to 94° C. (analysis, 73.15 per cent C, 9.5 per cent H), after recrystallization from hexane, behaves as a lactone (carbonyl absorption at 5.65 μ, in chloroform) yielding upon saponification an acid, m.p. 123 to 125° C. (analysis, C, 66.53 per cent; H, 9.63 per cent), and no other recoverable product.

Briareum asbestinum. Decolorization and recrystallization of the precipitate separating from pentane extracts of *B. asbestinum* yields a colorless solid with strong carbonyl absorption at 5.65 and 5.80 μ, melting at 120 to 127° C., from ether-pentane, and hexane, $[\alpha]_D^{31}$ $-135°$ (c = 0.516, chloroform).

Pterogorgia americana. Pentane extracts of this animal deposit a solid from which may be crystallized a compound showing absorption in the carbonyl and hydroxyl regions, and yielding an unsaturated hydrocarbon on distillation *in vacuo.*

Leptogorgia setacea. Pentane extracts of beach-washed specimens of *L. setacea* of uncertain age yielded a small amount (0.05 per cent) of insoluble material melting at 192 to 196° C. after recrystallization from pentane-chloroform. The most interesting feature of the infrared absorption curve was the presence of three maxima in the carbonyl region at 5.65, 5.72, and 5.85 μ.

Rhipidogorgia flabellum. Steam distillation of the sea fan from Bermuda yielded a yellow semisolid that inhibited growth of *Streptococcus pyogenes* and *Mycobacterium tuberculosis.*

Discussion

The crystalline lactones obtained thus far from the various species of gorgonians are all different compounds. Their function may be related to their weak antibiotic activity. Since these gorgonians have zooxanthellae associated with them, one must consider the possible role of these algal cells in the formation of the terpenoid compounds. We have not yet been able to examine the isolated zooxanthellae for their content of terpenoid compounds.

Acknowledgments

We express our appreciation to Paul R. Burkholder for testing some of our material for antibiotic activity, and to Esther M. Ciereszko for carrying out many of the extractions. We especially thank William H. Sutcliffe and Henry Kritzler for their help in connection with the field work.

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CHEMISTRY OF COELENTERATES. II. OCCURRENCE OF TAUROBETAINE AND CREATINE IN GORGONIANS*

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In the course of a study of the chemistry of gorgonians, we have isolated taurobetaine, $(CH_3)_3\overset{+}{N}CH_2CH_2SO_3^-$, from *Briareum asbestinum* (Pallas) in amounts exceeding 0.3 per cent of dry animal. Identity of the substance was established by degradation to trimethylamine and ethylene sulfonic acid, and by comparison of its infrared absorption spectrum with that of synthetic taurobetaine.

Examination of extracts of gorgonians by paper chromatography has shown creatine to be present in *Plexaura flexuosa* Lamouroux, *P. homomalla* Esper, *P. flavida* (Lamarck), *Plexaurella nutans* Duchassaing and Michelotti, *Plexaurella dichotoma* (Esper), *Pterogorgia acerosa* (Pallas), *Pterogorgia americana* (Gmelin), *Rhipidogorgia flabellum* (L.), and *Eunicea mammosa* Lamouroux. Three species, *Pterogorgia acerosa*, *Pterogorgia americana*, and *Rhipidogorgia flabellum* appeared to contain traces of glycocyamine, a precursor of creatine. No other guanidine bases were detected.

Methods and Results

Isolation and Identification of Taurobetaine

Briareum asbestinum was collected on rocky bottom near South Bimini. The freshly collected animals were dried in an oven at 60° C., crushed, and extracted successively with pentane, ether, and acetone. The powdered material was then extracted in a Soxhlet apparatus with methanol. After 3 hours, bumping occurred because of accumulation of sodium chloride. The hot extract was filtered, allowed to cool to room temperature, and chilled in a refrigerator overnight. From 660 gm. of *Briareum* 1.02 gm. of crystalline material was obtained after washing with methanol and ether. An additional 0.99 gm. was obtained by continuing extraction for 21 hours longer. The crystals were very soluble in water and were recrystallized from methanol, decomposing with evolution of gas at 344° C. (corr.) after insertion in the melting point block at 338° C., as did authentic taurobetaine prepared from taurine by action of diazomethane.[1] Analysis of the crystalline material from *Briareum* gave 35.45 per cent C, 8.68 per cent H, 8.24 per cent N and 18.90 per cent S; required for $C_5H_{13}NO_3S$: 35.91 per cent C, 7.83 per cent H, 8.37 per cent N and 19.17 per cent S.

A solution containing 0.2 gm. of the taurobetaine from *Briareum* and 0.266 gm. barium hydroxide in 25 ml. of water was boiled until evolution of trimethylamine (identified as the picrate and chloroplatinate) was complete. The

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solution was acidified (*p*H 2) with dilute sulfuric acid to remove barium ion. The filtered solution was evaporated to near-dryness, potassium carbonate solution was added to give an alkaline reaction (*p*H 8.5), and the solution was again evaporated. The residue was extracted with hot ethanol. Silky crystals of a potassium salt separated on cooling. The potassium salt, (0.047 gm.) m. p. above 350° C., was identified as that of ethylenesulfonic acid by conversion to the S-benzyl thiouronium salt, m.p. 142 to 144° C. Whitmore and Landau[2] give 145 to 146° C. as m.p. of S-benzyl thiouronium ethylenesulfonate. James[3] (see also Suter[4]) had reported formation of a salt of isethionic acid in the alkaline degradation of taurobetaine. We believe that in James' hands the ethylenesulfonic acid originally formed had been converted to isethionic acid by heating with excess sulfuric acid.[5] We avoided use of excess acid in working up our degradation mixture.

The infrared absorption spectra of synthetic taurobetaine and of the substance obtained from *B. asbestinum* were superimposable.

Creatine and Glycocyamine in Gorgonians

The method used in determining the nature of the guanidine bases present in the gorgonians was that of Roche *et al.*[6] Ten grams of the dried, ground cortex of each species was used for the analyses. Five volumes of 2 per cent acetic acid were added to each sample, and the mixture was heated for 5 min. on a boiling water bath. The samples were filtered, and the cooled filtrates were passed through Amberlite IRC50 cation exchange columns in the acid form. Each of the columns was washed with 200 ml. of water and the absorbed guanidine bases were eluted with 2 N ammonium hydroxide. The eluates were concentrated to near-dryness *in vacuo* and each was diluted with water to a volume of 2 ml. The samples were chromatographed on Whatman No. 1 paper, 0.05 ml. of each sample being used for a run. Five μg. of arginine and of creatine were run as standards. Six solvent systems[6] were employed. Each sample was run three times in each solvent to permit the papers to be developed by three different sprays, the Sakaguchi spray for arginine and other monosubstituted guanidines, a diacetyl-α-naphthol spray for creatine and other guanidines, and a ninhydrin spray to reveal the presence of arginine and other amino acids. Creatine was found in the gorgonians listed in the introduction.

Pterogorgia acerosa, *Pterogorgia americana* and *Rhipidogorgia flabellum* gave faintly positive Sakaguchi spots at the same R_f value as the creatine spot. It appears that glycocyamine has R_f values close to those of creatine; the positive Sakaguchi test plus the negative ninhydrin reaction suggest the possibility that there is a small amount of glycocyamine present along with the creatine.

DISCUSSION

The discovery of the occurrence of taurobetaine in *Briareum asbestinum* by us, and in sponges by Ackermann and List,[7] adds another derivative of taurine to the roll of natural products related to taurine. Lindberg[8] has found N-methyl taurine and di-N-methyl taurine in red algae, and has postulated that the trimethyl derivative, taurobetaine, should occur as an intermediate in the formation of choline sulfate that occurs in these algae. Taurine itself has a widespread occurrence in nature, and taurocyamine, asterubin, thiotaurine, and hypotaurine have also been found.

Whether taurobetaine plays an active role in metabolism, as in transmethylation, or as an intermediate in the formation of choline sulfate, as suggested by Lindberg, or of trimethylamine oxide and isethionate, which may be looked upon as possible degradation products of taurobetaine, remains to be seen. It may play a more passive role, as in regulation of osmotic pressure for which it may be ideally suited, since as a molecule that is at the same time a salt of a strong acid and of a strong base, it requires no counter ion.

Creatine was the only guanidine base found in the gorgonians, apart from traces of what appeared to be glycocyamine. Creatine phosphate thus may be the phosphagen in these animals. Roche *et al.* have reported finding traces of creatine together with arginine in the sea anemones, *Anemonia sulcata* Penn. and *Calliactis parasitica* Couch,[6] and in the alcyonarian, *Alcyonium palmatum* (Pallas),[9] but they found arginine alone in *Actinia equina* L., the only other coelenterate examined.

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PHOSPHAGENS OF MARINE ANIMALS

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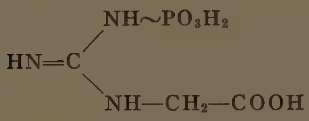
In the study of substances having biological activity in muscles, it may be noted that of the five phosphagens known at present, four are found in marine animals. This fact raises questions concerning the biological significance of these products.

Following the isolation of the first known phosphagen, creatinephosphate, by Fiske and Subbarow (1929) and of argininephosphate by Meyerhof and Lohmann (1928), systematic investigations were undertaken for other types of phosphagens. Because of the results obtained by two groups of workers (Arnold and Luck, 1933, Kurtz and Luck, 1937; and Baldwin and Yudkin, 1949), it was thought that unknown phosphagens, with lability different from that of arginine- and creatinephosphate, were present in marine worms. On the other hand, in our work on guanidine derivatives it was found that one single guanidine, either taurocyamine or glycocyamine (Thoai *et al.*, 1953; Thoai and Robin, 1954*a*), is present as such in the muscles of some marine worms even though it is found associated with arginine in the genital and intestinal tracts. This observation led to the isolation as barium salts of two new phosphagens, phosphotaurocyamine from *Arenicola marina* and phosphoglycocyamine from *Nereis diversicolor* (Thoai *et al.*, 1953). Similarly, it was found that lombricine or guanidoethylserylphosphate was the one guanidine present in muscle of the earthworm *Lumbricus terrestris*; this led to the isolation, as barium salt, of phospholombricine (Thoai and Robin, 1954*b*). The last-named phosphagen is the only one not found in marine animals (FIGURE 1).

The four phosphagens distributed among marine organisms can be isolated or synthesized as alkaline salts in crystalline form. The isolation of phosphagens is based on the relative solubility in water of their barium or calcium salts, much higher than that of inorganic phosphate, and on their insolubility in concentrated ethanol. In this way creatinephosphate was isolated by Fiske and Subbarow and argininephosphate by Meyerhof and Lohmann. With ion-exchange resins such as Permutit 50 or Dowex 50, barium or calcium ions can be replaced by alkaline ones; taurocyamine and glycocyamine phosphates were thus isolated as crystalline ammonium salts, free of guanidine derivatives that are always associated with phosphagens obtained by other methods (Thoai and Thiem, 1957).

The synthesis of these compounds is based on Zeile and Fawaz's (1938) method: phosphorylation of guanidine derivatives by $POCl_3$ in an alkaline medium. Using this technique, Ennor and Stocken (1948) prepared crystalline sodium creatinephosphate. Recently the process was simplified and the yield of sodium creatinephosphate doubled (Pradel *et al.*, 1959). With ion-exchange resins used during the purification, taurocyamine- and glycocyamine-phosphates were synthesized and crystallized as ammonium salts (Thoai and Thiem, 1957). Until recently argininephosphate could not be synthesized because several other phosphorylated compounds are produced simultaneously during the phosphorylation in alkaline medium, and the separation between them is not easy. Ennor and his collaborators (Ennor and Morrison, 1958; Ennor *et al.*, 1956) have attempted the synthesis by blocking the α-amino group as a copper complex, but they failed to prepare arginine phosphagen. With ion-exchange resins, the phosphagen can be separated from other phosphorylated compounds. In analytical and chromatographic investigations that have been carried out the synthetic product that crystallized as the lithium salt (95 per cent pure) appears to be identical with the natural phosphagen, isolated from scallops and *Arca noaea* (Thiem *et al.*, 1960).

Phosphagens can be identified even in small amounts of biological material, using paper chromatography. The presence of one single guanidine derivative in isolated muscle tissue indicates clearly that it gives rise to the corresponding phosphagen.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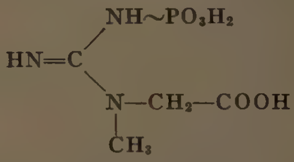
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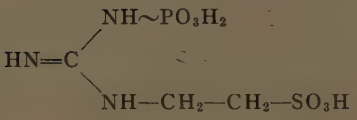
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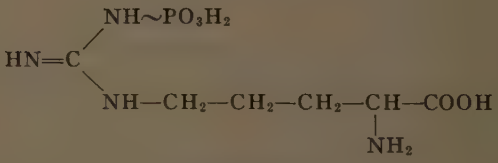
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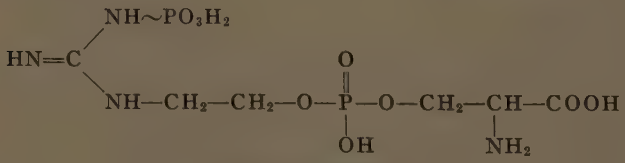
phospholombricine

FIGURE 1.

The most remarkable property of the phosphagens is their lability. These compounds are hydrolyzed easily by dilute acids. This lability of the phosphagens runs in parallel with their thermodynamic properties. At 30° C. with 0.1 N HCl, phosphocreatine is hydrolyzed twice as fast as phosphoarginine, and three times as fast as phosphotaurocyamine and phosphoglycocyamine (Pradel and Thoai, 1960), as shown in TABLE 1.

In the same order, free energy of hydrolysis decreases from phosphocreatine to phosphoarginine and phosphotaurocyamine. According to the equilibrium constant of transphosphorylation between the two former phosphagens and adenosine diphosphate (ADP), as determined by Lehmann (1936), the values calculated amount respectively to −11 and −8 kcal./mole at pH 9.8 (Oesper, 1950). We do not yet have data concerning phosphoglycocyamine, but the free energy of hydrolysis of phosphotaurocyamine has been calculated from the equilibrium constant of the reaction

$$\text{Ty}{\sim}\text{P} + \text{ADP} \rightleftharpoons \text{ATP} + \text{taurocyamine}$$

The value is −7 kcal./mole at *p*H 7.1 (Pradel and Thoai, 1960).

In fact, the differences between free energies of hydrolysis of the phosphagens should be smaller than were reported. According to the latest data concerning free energy of hydrolysis of ATP, −7 to −8 kcal./mole (Morales *et al.*, 1955; Benziger and Hems, 1956), and taking into account that free energy of hydrolysis of phosphocreatine is 1.1 kcal./mole above that of ATP, the value attributed to this phosphagen should be much smaller than has thus far been considered.

In all cases, free energy of hydrolysis of phosphagens is higher than that of ADP, thus favoring their biological activity. Since Lundsgaard's (1931) finding that phosphagen is used during muscular contraction, transphosphorylation reactions between phosphorylguanidines and ADP have been studied, using the general formula:

TABLE 1

FREE ENERGY OF HYDROLYSIS AND STABILITY OF PHOSPHAGENS

Phosphagens*	Free-energy hydrolysis (cal./mole)	Hydrolysis at 30° C. in 0.1 *N* HCl for 30 min. (%)
P∼Cr	−11.000	47.5
P∼Arg	−8.000	25.4
P∼Ty	−7.600	14.6

* P∼Cr = phosphocreatine; P∼Arg = phosphoarginine; and P∼Ty = phosphotaurocyamine.

$$\text{G}{\sim}\text{P} + \text{ADP} \underset{p\text{H } 8.5 \text{ to } 9.5}{\overset{p\text{H } 6.8 \text{ to } 7.1}{\rightleftharpoons}} \text{ATP} + \text{guanidine derivatives}$$

where G∼P = creatine-, arginine-, taurocyamine-, glycocyamine,- and lombricinephosphates.

Some of the enzymes responsible for these reactions have been highly purified and crystallized: creatinephosphokinase (Kuby *et al.*, 1954) and argininephosphokinase (Elodi and Szorenyi, 1956). These are obtained from terrestrial vertebrates and fresh-water invertebrates, but since Lehmann's (1936) work on octopus muscle, no data have been reported from marine animals. Baldwin and Needham (1937), Morrison *et al.* (1957), Yudkin (1954), and Hobson and Rees (1957) have focused their work on the biological distribution of various kinases. Until quite recently, Morrison *et al.* (1957) have attempted to purify argininephosphokinase from the sea crayfish (*Jasus lalandi* and *Jasus verreauxi*) and they have determined the properties of the enzyme. From *Arenicola marina* muscle, taurocyaminephosphokinase has been purified (Thoai and Pradel, unpublished work).

Muscles from marine worms, excluding genital and intestinal tracts, are homogenized and extracted twice with water. After centrifugation, the supernatant is fractionated, the first stage with crystalline ammonium sulfate and the second and third stages with saturated saline solution. The latter precipitate, dissolved in glycine buffer, is treated first with calcium phosphate gel, and then with alumina $C\gamma$ gel. The adsorption is negative and the final supernatant is fractionated with ethanol at −15° C. The taurocyaminephosphokinase thus prepared shows a turnover of about 300 mmole of phosphate transferred per minute per gram of protein. The figure is similar to that of creatinephosphokinase studied by Kuby *et al.* (1954). The highly active prep-

TABLE 2
BIOLOGICAL DISTRIBUTION* OF PHOSPHOGENS†

Zoological groups	P∼Cr	P∼Arg	P∼Ty	P∼Gly	P∼Lom
Poriphera	+	+			
Coelenterata		+			
Platylhelminthes		+			
Sipunculoidea		+	+		
Annelida					
Polycheta	+	+	+	+	
Oligocheta					+
Arthropoda		+			
Mollusca		+			
Echinodermata					
Asteroidea		+			
Crinoidea		+			
Echinoidea	+	+			
Holothuroidea	+	+			
Ophiuroidea	+	+			
Chordata	+				
Vertebrata	+				

* Ennor and Morrison (1958).

† P∼Cr = phosphocreatine; P∼Arg = phosphoarginine; P∼Ty = phcsphotaurocyamine; P∼Gly = phosphoglycocyamine; and P∼Lom = phospholombricine.

TABLE 3
BIOLOGICAL DISTRIBUTION* OF ARGININE, CREATINE, AND TAUROCYAMINE IN MUSCLES AND GENITAL GLANDS OR CELLS

Species	Muscles	♀	♂	O〰
Sphaerechinus granularis Lmk.	A C	A		
Martasterias glacialis L.	A	A		C
Leptosynapta inhaerens M.	A			C
Maia squinado R.	A	A	A	
Arenicola marina L.	Ty			C
Audouinia tentaculata	C			C
Dasybranchus caducus G.	C			C
Ascaris lumbricoides L.	A	A	A	
Calliactis parasitica C.		A		C

Key: A = arginine; C = creatine; Ty = taurocyamine; ♀ = female glands; ♂ = ameboid sperm cells; and O〰 = ciliate sperm cells.

* Data from Greenwald (1946); Mende and Chambers (1953); and Roche *et al.* (1957).

aration is homogenous to Tiselius electrophoresis. It is 95 per cent pure (Thoai and Pradel, unpublished work).

With taurocyaminephosphokinase the equilibrium constant K_e of the reaction

$$\mathrm{Ty{\sim}P} + \mathrm{ADP} \rightleftharpoons \mathrm{ATP} + \text{taurocyamine}$$

has been determined. At 26° C., pH 7.1, the mean value of K_e amounts to 0.55. Thus, free energy of the reaction was calculated $\Delta G = -360$ cal./mole (Pradel and Thoai, 1960). This value is near the heat (-350 cal./mole) liberated in the differential microcalorimeter during the same reaction (Thiem and Thoai, unpublished data).

The next question to be considered in the study of the phosphagens is a possible connection between the variety of phosphagens and their biological significance. For a long time, according to an ontogenic interpretation, it was thought that creatinephosphate characterized vertebrates and that argininephosphate characterized invertebrates. It is evident today that the situation is more complex.

From TABLE 2 it can be seen that except for phospholombricine, all phosphagens are present in marine animals. Creatinephosphate is found not only in vertebrates, either terrestrial or marine, but also in many other zoological groups: Porifera, Annelida, Echinoderma, Holothuroidea, Ophiuroidea, and Chordata. Phosphoarginine is as widespread in the invertebrates as phosphocreatine is in the vertebrates. It must be stressed that marine worms offer the greatest variety of phosphagens: in one group, the Polycheta, we find four of them: creatine-, arginine-, taurocyamine-, and glycocyaminephosphates.

Of course, the particular distribution of these compounds may be explained by variations in metabolism, due partly to the stage of evolution and partly to ecological conditions. However, other data may be mentioned for a better understanding of the biological significance of phosphagens.

As is shown in TABLE 3, animals that have a certain type of phosphagen in their muscles are likely to possess another type of product in the genital glands or cells. Whatever the single guanidine derivative in muscles (arginine, creatine, or taurocyamine), arginine is present only in female glands or ameboid sperm cells, and creatine in ciliate sperm cells.

However, no definite conclusion can be formulated because the available data are still very fragmentary. It may be suggested that to each type of muscle should be adapted a phosphagen; the compounds of high thermodynamic potential should be used for fast muscles and those of lower potential for slow muscles.

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HISTOENZYMOLOGICAL DATA ON THE DIGESTIVE TRACT OF *OCTOPUS VULGARIS* LAMARCK (CEPHALOPODA)

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Examination of the mode of nutrition in cephalopods has been of interest to numerous investigators since Swammerdam (1757); Milne-Edwards (1859) refers to a dozen or more works relative to the morphology and function of the digestive tract in mollusks.

Acidity and alkalinity of the secretions elaborated by morphologically-distinct segments of the digestive tract were characterized by the end of the 19th Century. Claude Bernard (1856) estimated that the saliva of Cephalopoda was alkaline, as was the gastric secretion; as for the intestinal secretion, it was sometimes alkaline and sometimes acid, correlating with the presence or absence of nitrogenous matter in the food. Krukenberg (1878), Griffiths (1884), and Cohnheim (1902*a* and *b*) estimated that the intestinal secretions of *Eledone* and *Sepia* were alkaline. However, Bert (1867), with *Sepia*, and Frédéricq (1878) and Jousset de Bellesme (1879), with *Octopus*, were not of that opinion; they estimated, respectively, that the digestive secretions were "constantly," "frankly," and "strongly" acid, while Bourquelot (1885) and Henri (1903) found them "feebly" acid. The most significant investigations on the question are those of Falloise (1906); in *Eledone* and in *Octopus* this worker injected orally, with a thin catheter attached to a Pravaz syringe, a small quantity of a litmus solution; he saw that the solution remained blue in the crop and then became red in the stomach, the caecum, and the intestine.

Aside from the question of acidity some investigators have studied the modifications undergone by certain substrates when in contact with extracts of tissues and the products of maceration (*macêrats*), just as, more rarely, (Krause, 1895, 1897; Ghiretti, 1953), when in contact with the action of pure digestive secretions obtained with a catheter. More often, investigators have worked with mixtures of secretions: caecal, gastric, "hepatic," and "pancreatic." Bidder (1950) said that "it is clear that careful examination of liver and pancreas extracts is needed, as well as of caecal and gastric juice and caecal extract, all at known stages of digestion." The difficulty of surgical intervention in Cephalopoda caused Bidder to add: "The answer to this problem is to be sought by the methods of histochemistry." It seems that this work has not been undertaken.

The investigations of Erspamer and his collaborators, however, have revealed, among other interesting data, (Vialli and Erspamer, 1938; Vialli, 1946) some essential biochemical differential characters bound to the species of Cephalopoda studied. Examining with great accuracy, by chromatography, the amines contained in the posterior salivary glands, Erspamer and Boretti (1951) demonstrated that the data obtained on one octopod could not be applied a priori to another octopod and, therefore, the data obtained on one Cephalopod cannot be generalized and applied to all Cephalopoda. Thus, for example, the extracts of the posterior salivary gland of *Octopus macropus* and of *O. vulgaris* are rich in tyramine, while those of *Eledone moschata* are lacking in that amine; the posterior salivary gland of *Octopus macropus* contains histamine, while that of *O. vulgaris* contains none.

The present study, therefore, is limited to *Octopus vulgaris* Lamarck (Naef, 1923).

Examination of the glandular complex that is called "Mitteldarmdrüse" or "glande digestive" in the mollusks, which is appended to the digestive crossway between the stomach, the caecum, and the intestine of *Octopus vulgaris*, reveals a remarkable duality. This formation is composed of two glands contained in the same capsule. The duality of this formation was observed as early as 1826 by Blainville, who distinguished "un petit espace ovale où il y a une couleur plus blanche que le reste."* Müller (1853) and Brock (1880) made the same observation; but in 1882 Vigelius observed that "die höchst spärlichen Angaben, welche die Literatur über die Anhänge der Lebergänge bei den Cephalopoden gewährt, sind so kurz und zugleich so oberflächlich, dass sie kaum Erwähnung verdienen"† (see also Vigelius, 1881, 1883).

Livon (1881), in his study of the physiology of digestion of *Octopus*, expressed the idea that we must see "une glande speciale" in the "masse jaune" at the inferior face of the "liver"; almost at the same time Vigelius (1882) distinguished this mass under the name of "glandular zone." Griffiths (1884), Bourquelot (1885), Frenzel (1886), and Henri (1903) have tried to ascertain if the "Mitteldarmdrüse" of Cephalopoda was "hepatic," "pancreatic," or "hepato-pancreatic," without paying much attention to its heterogeneity. In examining the "Mitteldarmdrüse" of *Octopus vulgaris* Lamarck, one realizes how inadequate the names liver and pancreas are; the two precaecal glandular formations thus named have no analogy at all to the liver and pancreas of the vertebrates. In both cases it is a matter of glands in tubes (FIGURE 1), in which the histochemical characters are quite different. The most important glandular mass in size is anterior, and elaborates several complex secretions; in the posteroinferior zone of this mass one finds the "Drusenkorper" of Müller (1853), or the "Drusenterritorien" of Brock (1880), which seems to elaborate a homogeneous secretion. The secretions elaborated by the anterior gland pass through the little posterior gland, so that the excretory channels of this precaecal complex transport to the caecum mixed secretions (FIGURE 1). This fact, observed by Livon (1881) and Vigelius (1882), invalidates all the biochemical observations made on the so-called "hepatic" secretion, even when, in the best of cases, it has been collected with a catheter in the excretory channel near its opening into the caecum (Henri, 1903). The name "glande médio-intestinale" is not adequate to designate the precaecal complex of *Octopus*, because this gland is not joined to the middle of the intestine and, in addition, because this name does not give the slightest indication of the duality of the glandular complex in question.

The name "glande digestive" is no longer adequate, since it does not even

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* "A small oval space that has a color whiter than the rest." (Editor's translation.)

† "The most informative accounts given in the literature on the connections of the hepatic duct in Cephalopoda are so short and, in some instances, so superficial, as hardly to deserve recognition." (Editor's translation.)

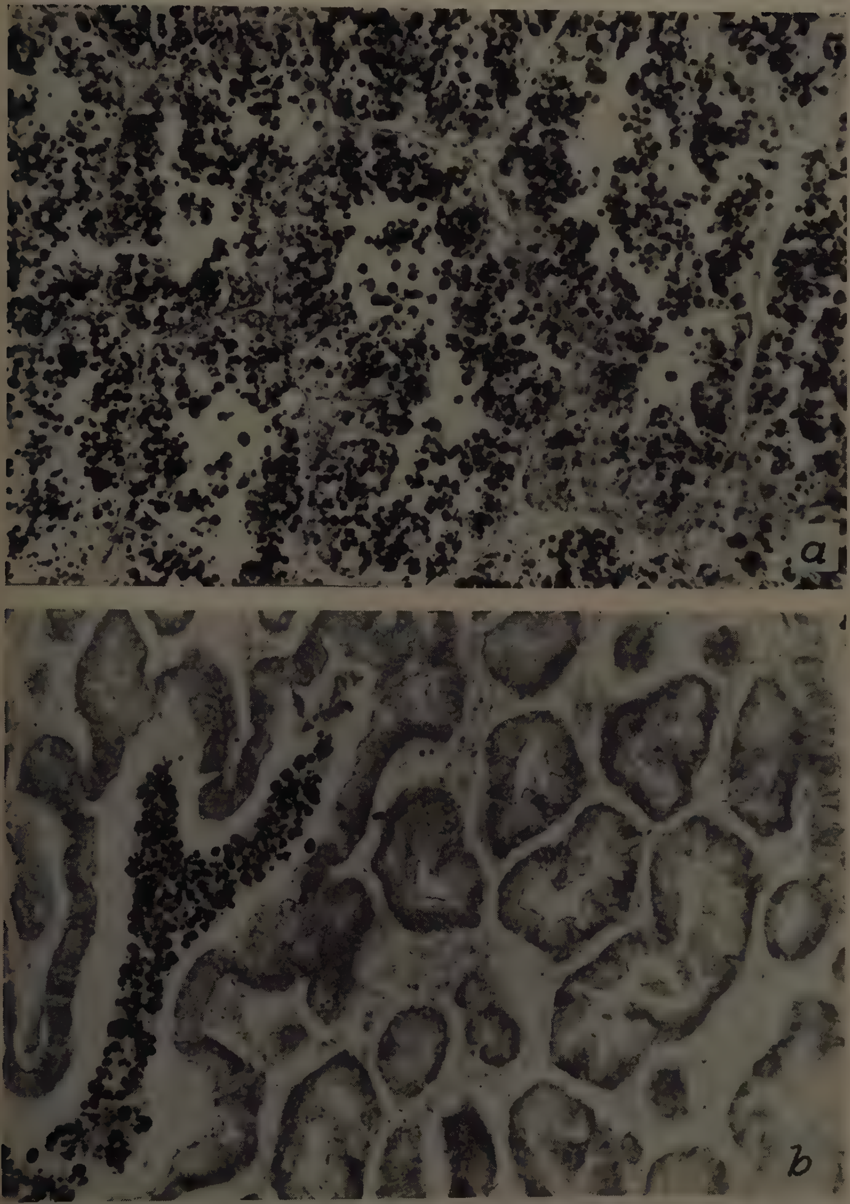

FIGURE 1. *Octopus vulgaris* Lamarck after formaldehyde fixation and staining by the Gabe and Martoja-Pierson technique. (*a*) A section of the anterior caecal gland (so-called "Mitteldarmdrüse" or "digestive gland"). (*b*) A section of the posterior caecal gland (so-called "Drusenkorper" or "Drusenterritorien" of Müller [1853] and Brock [1880], respectively). The products elaborated by the anterior caecal gland pass through the posterior caecal gland.

point out that it is a matter of one salivary gland as distinct from another. One of these glands, the largest in volume, is anterior in relation to the other, and has characteristics similar to those of the "digestive gland" or "gland médio-intestinale" of other mollusks. It can be designated the anterior caecal gland; the other, adjacent to the posterior part of the preceding gland, has no equivalent, in so far as is known, in other mollusks, and may be called the posterior caecal gland. It is under these names that I shall discuss some of the histoenzymological characteristics of the two glands that comprise the precaecal complex.

MATERIALS AND METHODS

The present observations are based on material from five specimens of *Octopus vulgaris* Lamarck, three of which came from Biarritz and were studied under excellent conditions in the marine laboratory at Arcachon in November, 1959; the remaining two specimens came from Roscoff and were examined at at Jouy-en-Josas in August, 1959. The tissues of the two groups of *Octopus* did not differ significantly.

Aside from the oxidase activities, which were detected on fresh digestive tract divided longitudinally into two equal parts and submitted to different substrates, the esterase activities were detected on tissues fixed during a short period of time, always less than 24 hours, by 10 per cent neutralized formaldehyde; they were cut after freezing and subjected to various baths. The phosphatase activities were detected by modifications of the Gomori techniques (by using Gomori-Lillie for the alkaline phosphomonoesterase and Rabinovitch-Andreucci for the acid phosphomonesterase activities), and by the Wachstein-Meisel technique for the 5-nucleotidase and adenosine-triphosphatase activities. The carboxylic esterases were detected with the reactions of Burstone and of Koelle-Gerebtzoff. The cytochromoxydase, succinodeshydrogenase, and aminoxydase activities were detected, respectively, by the Nadi reaction, the Farber-Louviere reaction, and by a personal technique (1958) using tyramine, tryptamine, or histamine, and testing each one for aminoxydase activity.

Subsequently, the polysaccharides were revealed by the MacManus reaction and by staining with Alcian blue; the lipids were stained by Sudan black and by polychrome in one step (Arvy, 1958); the SH-groups were detected by the Adams technique; and the topography was ascertained by staining the basophilic substances with toluidine blue and by the trichrome staining of Gabe and Martoja-Pierson (1957).

OBSERVATIONS

Oxydase Activities

The oxydase and succinodeshydrogenase activities were detected on fresh digestive tract that had been removed whole and longitudinally divided into two equal parts; each half was treated with a different substrate.

The Nadi oxydase activity is weak, except in the masticatory muscles or muscular tunics of the crop and of the gizzard. The salivary glands and the intestine, when immersed in the Nadi reagent, do not become blue or, if they do, the reaction is weak and delayed. The caecal glands do not oxidize the Nadi reagent.

The free monoaminoxydase, tyraminoxydase, and tryptaminoxydase activities are not important in the digestive tract of *Octopus vulgaris*; only the anterior caecal gland has a strong tryptaminoxydase activity.

The histaminoxydase activity is the weakest of all those observed; only the posterior caecal gland and the posterior salivary glands have a noticeable histaminoxydase activity.

The succinodeshydrogenase activity, on the other hand, is comparatively strong; both the anterior and the posterior salivary gland, especially the latter, rapidly produce formazan upon contact of the Farber-Louviere bath.

Esterase Activities

The salivary glands. The weaker esterase activity of the anterior salivary glands, whether phosphatase or carboxylic esterase, usually is sufficient to differentiate them from the posterior salivary glands.

Of all the phosphatase activities that have been detected, the alkaline and the acid phosphomonoesterase activities are the strongest; they are absent in the intertubular tissue and exist in all the epithelial cells at the beginning of their secretory phase, when the cells are still rich in basophilic substance (FIGURE 2*a*). The phosphatase activity diminishes as the epithelial cell becomes full of secretory products. The epithelium of the collecting channels and the excretory channels lacks histochemically detectable phosphatase activity.

The adenosine-triphosphatase activity is relatively strong in the secretory epithelial cells of the posterior salivary glands and is confined to the basal zone of the cells.

It appears that histochemically detectable butyrylthiocholinesterase and acetylthiocholinesterase activities do not exist in the anterior salivary gland. On the other hand, studies of these two enzymatic activities reveal some interesting aspects of the posterior salivary gland. With butyrylthiocholinesterase as a substrate, at *p*H 6.8, the entire system of secretory tubules appears, with its particular mode of ramification and torsion. Cutting sections tangent to the tubules permits one to see that the secretory tubes and the excretory channels are surrounded by a network of tight, threadlike cells rich in enzyme (FIGURE 3). Using acetylthiocholine at the same *p*H reveals that the secretory tubules and the excretory channels are surrounded by a network of extremely attenuated, threadlike membranes that hydrolyze the ester (FIGURE 2*b*).

The acetylnaphtholesterasic activity of the posterior salivary gland has been found to be very strong and selectively localized in the intertubular connective tissue (FIGURE 2*c*). When immersed in the Burstone substrate the sections quickly became reddish and, upon the termination of the reaction, the intertubular connective tissue was deep purple, the base of the secretory epithelium of the tubules was also purple, the epithelium of the excretory channel was pink, and the mucus inclusions were colorless.

The anterior salivary gland gives only a slight hydrolyzing reaction to alpha-

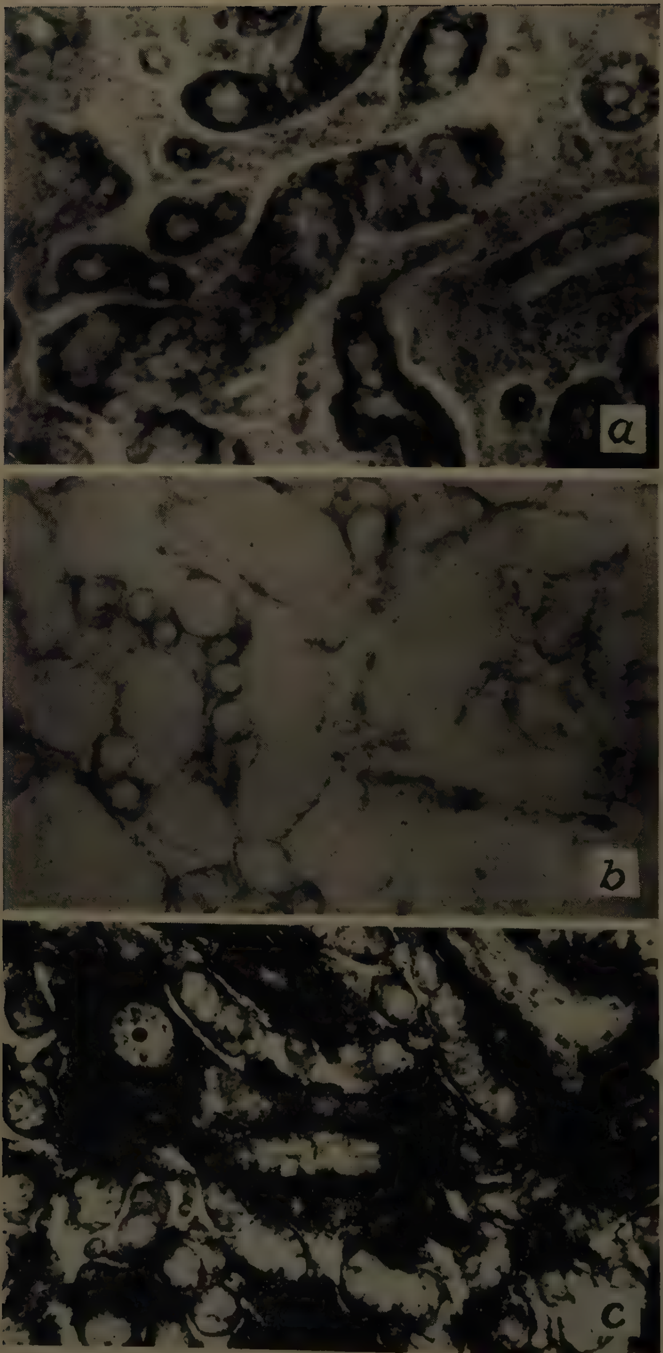

FIGURE 2. Posterior salivary gland of *Octopus vulgaris* Lamarck. (*a*) Basophilic substance stained by toluidine blue; (*b*) acetylthiocholinesterase activity; and (*c*) acetylnaphtholesterase activity.

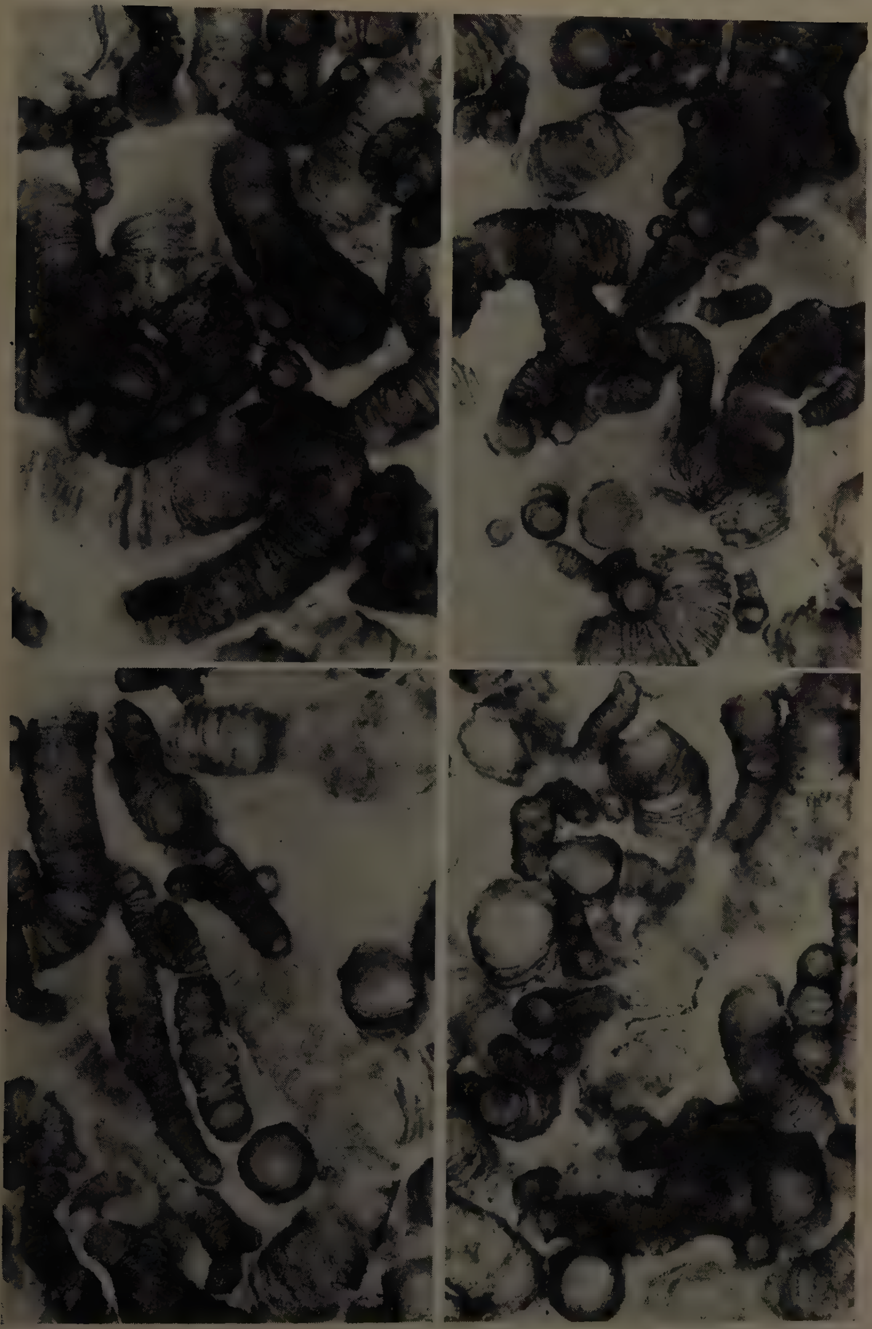

FIGURE 3. Posterior salivary gland of *Octopus vulgaris* Lamarck, showing butyrylthiocholinesterase activity.

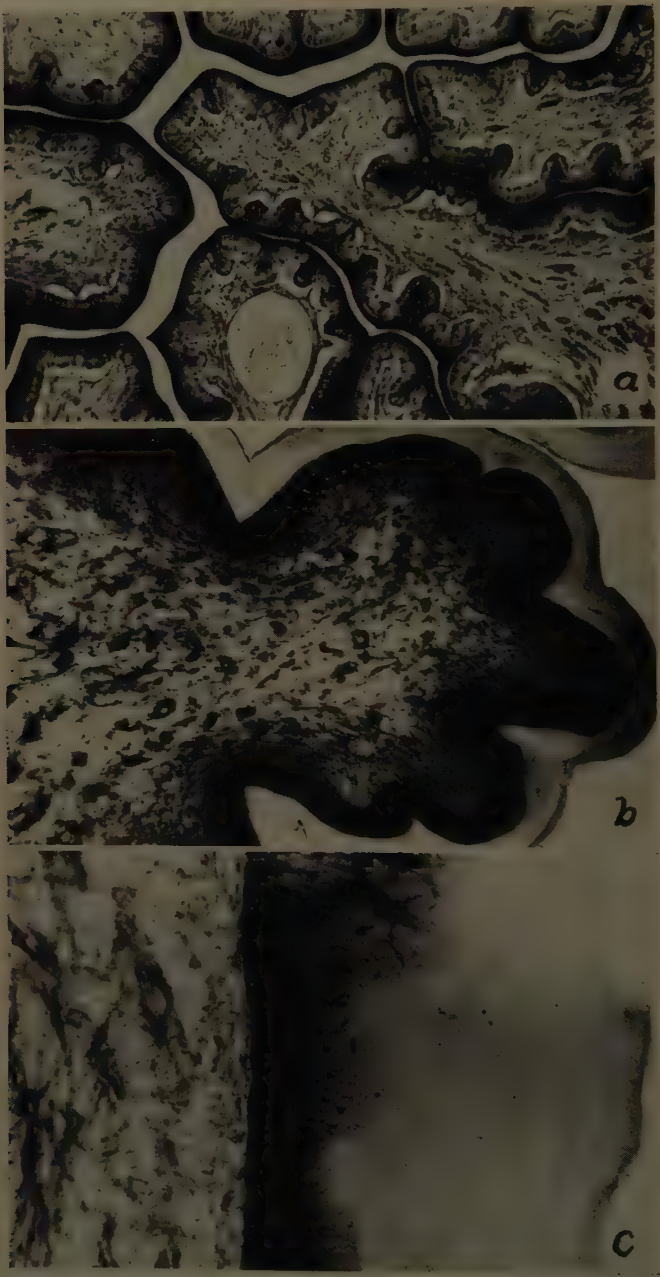

FIGURE 4. Various aspects of the cuticle in different regions of the digestive tract in *Octopus vulgaris* Lamarck. (*a*) Esophagus after staining of the cuticle with Janus green and of the nuclei with fast red; (*b*) segment near the gizzard; and (*c*) gizzard, after staining with toluidine blue.

naphthol acetate; the enzymatic activity is limited to the intertubular connective tissue and to the secretory granules of the apical pole of the epithelium.

The keratinized segment of the digestive tract. This entire segment, from the esophagus to the caecum, as well as the terminal intestine, is covered by a cuticle that is relatively thin in the esophagus but very thick and stratified in the gizzard (FIGURE 4*a*).

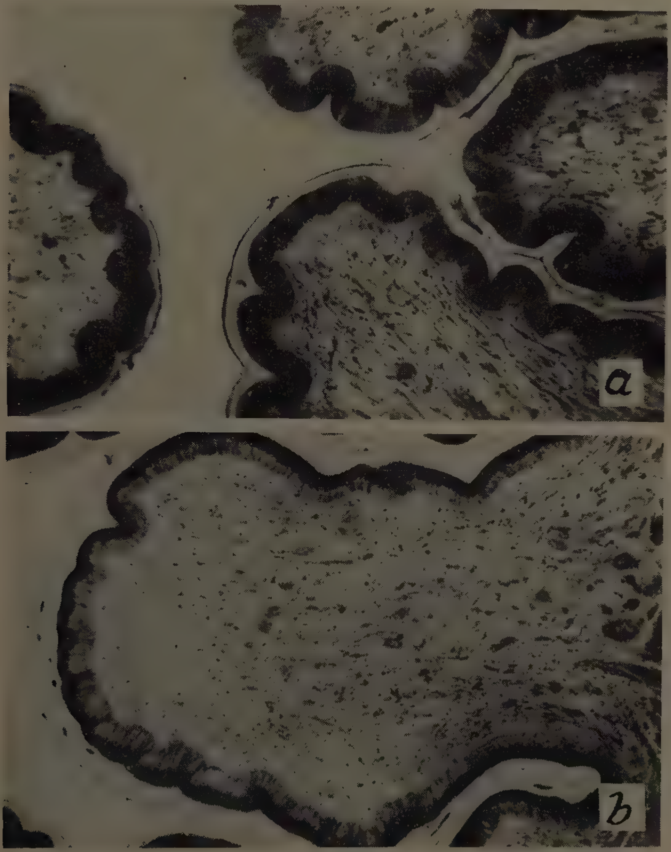

FIGURE 5. Comparison of the phosphomonoesterase alkaline activity (*a*), and of the adenosine-triphosphatase activity (*b*) in the esophageal epithelium.

The epithelium immediately beneath the keratinized cuticle consists of very narrow and high cells, with oval nuclei situated in the middle of these cells. The cells are rich in basophilic substance and have uniformly distributed and relatively weak (FIGURES 5*a* and 6*a*) alkaline phosphomonoesterase, 5-nucleotidase, and acetylnaphtholesterase activity. Comparatively, these epithelial cells have a strong acid phosphomonoesterase and adenosine-triphosphatase activity (FIGURE 5*b*); they predominate beneath the cuticle at the apical tiers of the cells.

The spiral caecum and the nonkeratinized segment of the digestive tract. It is only from the point where the digestive tract loses its subepithelial cuticle that the digestive mucous membrane of *Octopus* acquires esterase activities that are similar to those of the digestive tract vertebrates. The transition of keratinized epithelium to epithelium with a free apex in the intestinal lumen is not progressive, it is sudden: one cell has a cuticle and the next one has not. Beyond this last cell the acid phosphomonoesterasic and acetylnaphtholesterasic activities tend to predominate in the apical pole of the cells (FIGURE 6*b*).

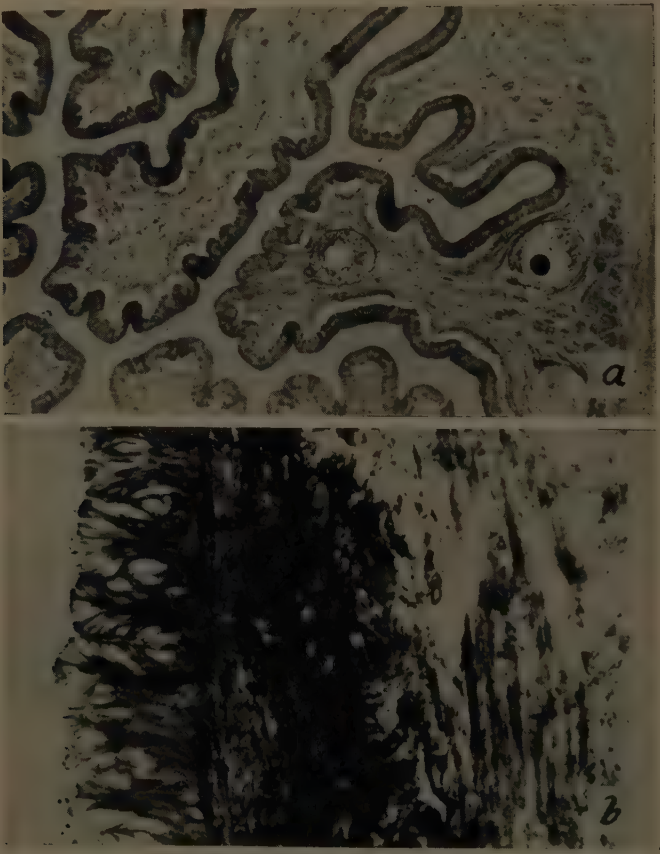

FIGURE 6. Comparison of the acetylnaphtholesterase activity in the esophageal epithelium (*a*), and in the spiral caecum (*b*).

All along the digestive tract, the muscles strongly hydrolyze the acetylcholine and butyrylcholine (FIGURES 7*a* and *c*). Here one cannot distinguish myoneural synapsis, similar to that of the vertebrates, but the subepithelial zone of the mucous membrane contains ganglia with many cells characterized by pericaryones rich in acetylthiocholinesterase (FIGURES 7*b* and *c*).

The precaecal glandular complex. The two glands, anterior caecal and posterior caecal, are equally rich in acetylnaphtholesterase (FIGURE 8*a*), but they differ in all other histochemically detectable enzymatic activities that have been ob-

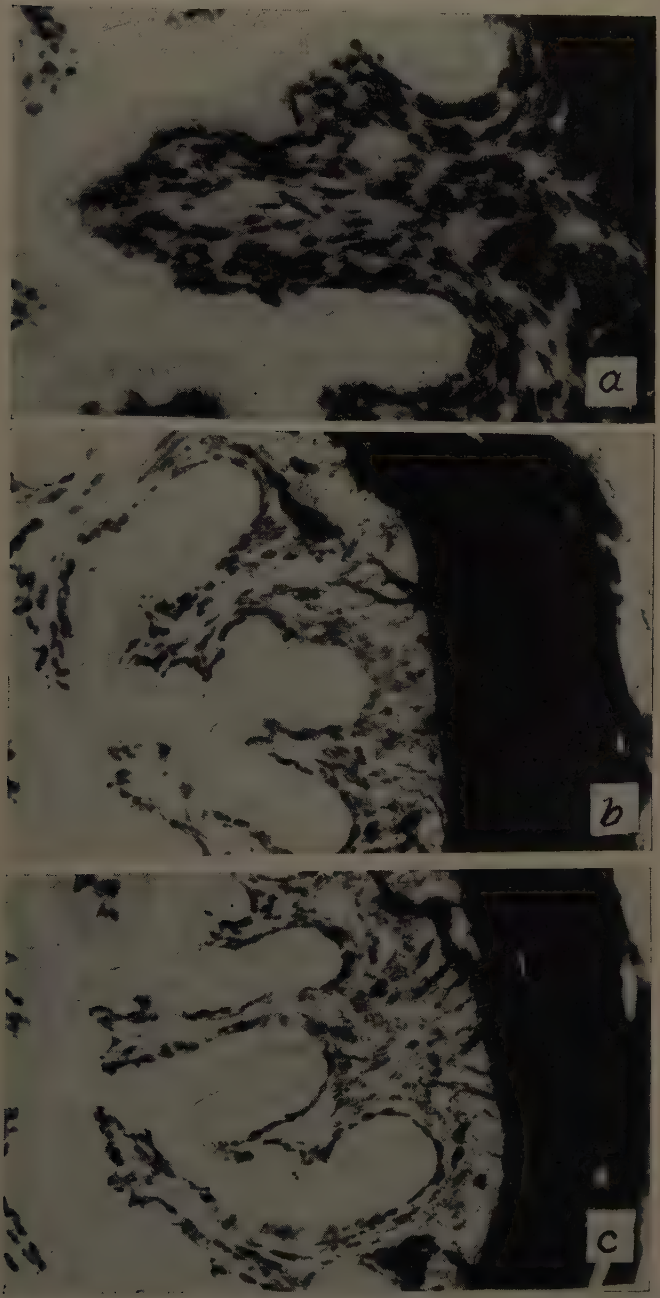

FIGURE 7. Aspect of sections of the intestine of *Octopus vulgaris*, after detection of the butyrylthiocholinesterase activity (*a*), and acetylthiocholinesterase activity (*b* and *c*).

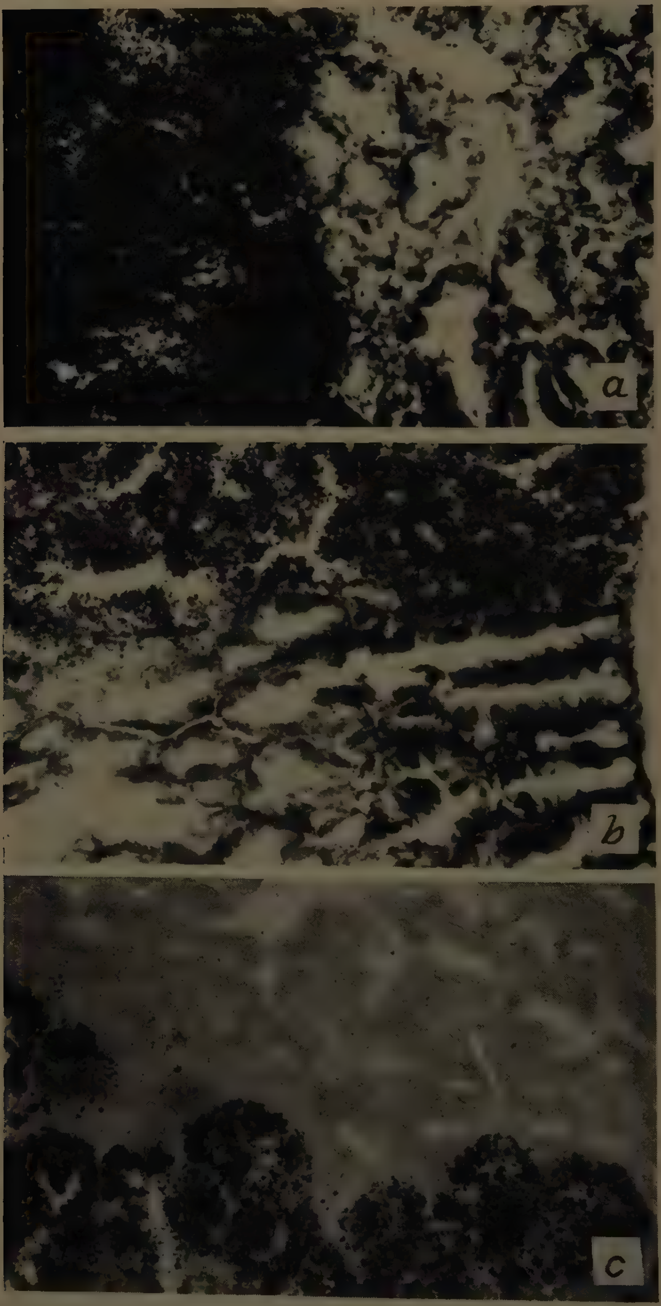

FIGURE 8. Precaecal glandular complex in *Octopus vulgaris* Lamarck, after detection of the acid phosphomonoesterase activity (*a*), acetylnaphtholesterase activity (*b*), and butyrylthiocholinesterase activity (*c*).

served here. The anterior caecal gland is rich in alkaline phosphomonoesterase and still richer in acid phosphomonoesterase, while the posterior gland is deficient in alkaline phosphomonoesterase and has acid phosphomonoesterase activity only in the basal part of its tubules and in the epithelium of the excretory channels. In the epithelium the acid phosphomonoesterase activity has a double polarity: this activity predominates in the basal and apical parts of the epithelial cells. Similarly, the anterior caecal gland is rich in butyrylthiocholinesterase, while the posterior caecal gland lacks that enzyme (FIGURE 8*c*). Finally, the posterior caecal gland (FIGURE 9), in contrast to the anterior gland (FIGURE 10), possesses a peritubular net of fibers capable of hydrolyzing acetylthiocholine. It is only at the level of the blood vessels that the anterior caecal gland contains some nerve fibers that hydrolyze acetylthiocholine.

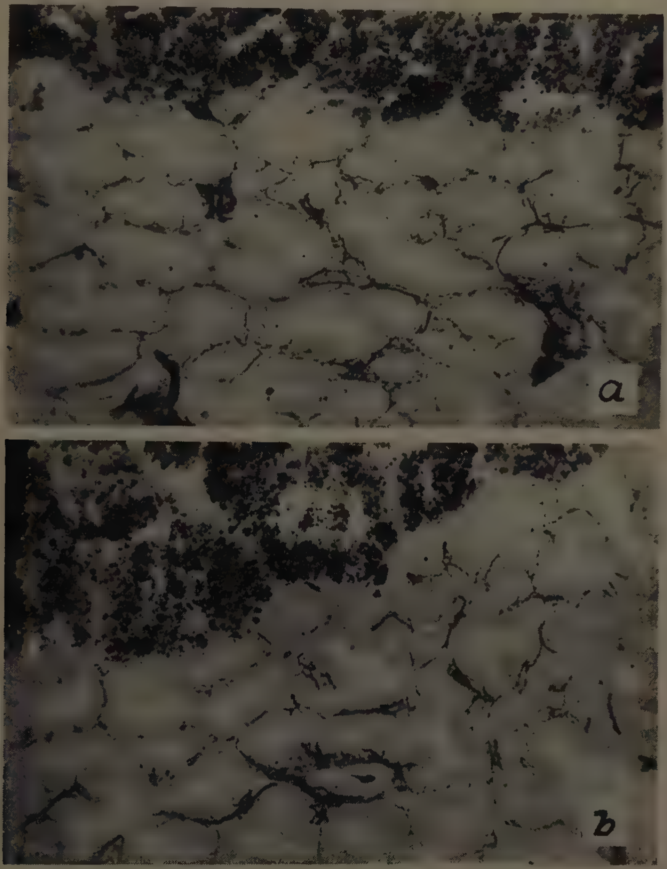

FIGURE 9. Two aspects of the posterior caecal gland of *Octopus vulgaris*; the acetylthiocholinesterase activity is restricted to a network of peri- and intertubular fibers.

Examination of the polysaccharides, lipids, and SH-groups shows no fundamental differences between the two glands constituting the precaecal glandular complex; both are rich in polysaccharides, lipids, and SH-groups.

Discussion

The early research on the digestive enzymes of *Octopus* has only historical value (Bourquelot, 1882, 1903; Enrique, 1902; Henze, 1913; and Verne, 1922). However, the most recent studies of Capurro and Zaccheo (1953, 1958), Gerebtzoff (1956), and especially those of Blaschko and his pupils have more interest for the histoenzymologist of today. The findings I have just summarized can be discussed from several points of view.

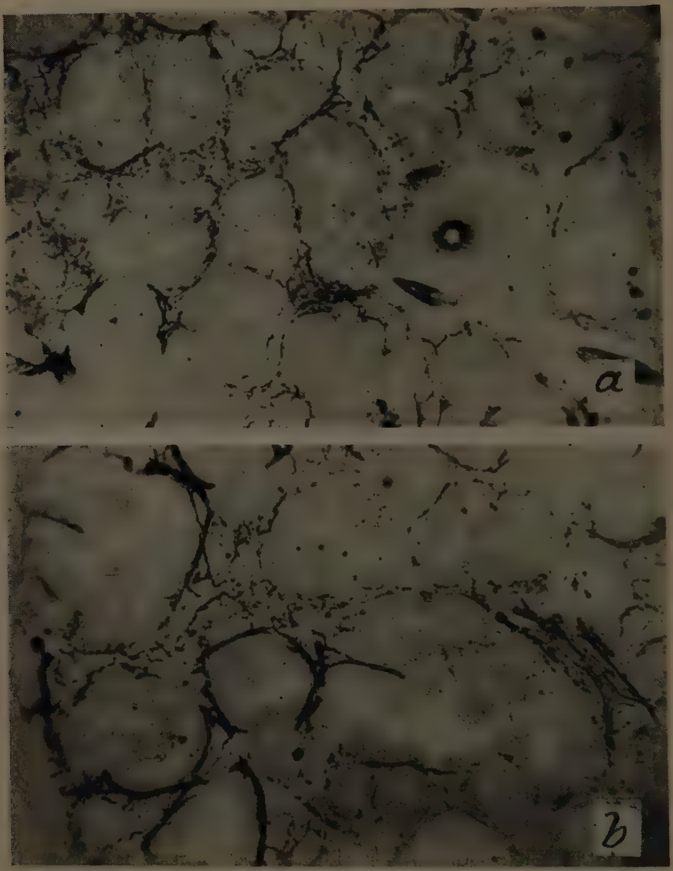

FIGURE 10. Two aspects of the caecal gland, anterior (*above*) and posterior (*below*) after detection of the acetylcholinesterase activity.

The Posterior Salivary Gland

Blaschko and Himms (1954) have drawn attention to the intensity of the aminoxidase reaction of the accessory digestive gland of Cephalopoda. The homogenates of the posterior salivary gland exhibited tyraminoxidase, tryptaminoxidase, and 5-hydroxytryptaminoxidase activities (Blaschko and Hawkins, 1952; Blaschko and Philpot, 1953); the wealth of tyraminoxidase and isoamylaminoxidase found in the anterior salivary glands of *Octopus* is about one half that of the posterior salivary glands (Blaschko and Hawkins, 1952). The tryptaminoxidase activity visible in thick or frozen sections of the posterior salivary gland in *Octopus* corresponds to the enzymatic activity that has been observed by Blaschko and his collaborators in the homogenates of the gland. The biological significance of the amines and of the salivary aminoxidase activity *in situ* remains to be explained. Two hypotheses have been expressed: Sereni (1930) considered the salivary amines as hormones, and held that in this case the salivary aminoxidase could intervene as factors in the hormonal regulation; Blaschko and Hawkins (1952), however, considered the aminoxidases as factors in the synthesis of the salivary amines. No single observation corroborates or discredits this latter interpretation.

The entire anterior segment of the digestive tract of *Octopus* seems to serve only for transport, massage (*malaxation*), and grinding of the food; its muscular tunics are powerful, its mucosa does not contain any glands, and its epithelium is covered by a cuticle that isolates it from the ingesta. Hence the enzymatic activities attributed to this segment by early investigators can be due only to secretions delivered at the tract's origin by the four salivary glands, or to secretions "backing up" from the spiral caecum and intestines toward the gizzard.

The digestive tracts of Octopoda are poor in leukocytes, and not one author has considered that these cells can play an important role analagous to that described by several investigators for the much larger number of leukocytes in digestion and assimilation processes in *Lamellibranchiata*. It seems that in *Octopus*, digestion and absorption are accomplished in the lumen and in the epithelium of the digestive tract.

The role of the salivary gland is even more enigmatic. Four different opinions have been expressed. Some authors (Bert, 1867; Jousset de Bellesme, 1879; Joubin, 1887; Rawitz, 1892; Krause, 1897; Falloise, 1906; and Craifaleanu, 1916, among others) have said that the gland has a digestive role; some others have denied a contribution in any manner to the digestive process (Frédéricq, 1878; Bourquelot, 1885; and Rouville, 1910). Still other authors considered them as excretory organs (Krause, 1897; Henze, 1905; and Cuénot, 1905), but the majority of workers see them as defensive organs or venom glands, necessary for the capture of prey because of their neurotropic toxins (Krause, 1895 and 1897; Lo Bianco, 1899; Briot, 1905; Livon and Briot, 1905 and 1906; Henze, 1905; Baglioni, 1909; Bauer, 1909; Rouville, 1910; Kruger, 1929; Erspamer, 1948; Ghiretti, 1953; Berry and Halstead, 1954). That the secretions of the salivary glands of *Octopus* are toxic for many vertebrates is not doubted (Marco, 1937). However there exists not one argument that permits a denial of digestive power to their secretions. On the contrary, Bacq *et al.* (1952) and Ghiretti (1953) found in these secretions a proteolytic activity and Romanini (1952, 1954) found a hyaluronidase activity. Romanini has demonstrated the existence, in the anterior as in the posterior salivary glands, of a factor endowed with an intense mucinolytic activity. Thus the extract of the salivary glands of *Octopus vulgaris* Lamarck, *O. macropus* Risso, and *Eledone moschata* digests Wharton's jelly: the metachromatic substance of the umbilical cord and the ground substance of the tracheal cartilage in the rat.

Moreover, histochemistry permits detection, in the anterior salivary gland as well as in its excretory channels, of a serous secretion that has an acetylnaphtholesterase activity.

It seems that the posterior salivary gland secretes several products; in effect, some tubules, or some parts of the tubules (FIGURE 2*a*), are remarkably deficient in basophilic substance. Perhaps this phenomenon represents different stages of the elaboration of the same product.

The presence of elements capable of hydrolizing acetylcholine and especially butyrylcholine in the tubules of the posterior salivary glands of *Octopus vulgaris* Lamarck perhaps may be connected with the observations of physiologists (Hyde, 1897; Botazzi, 1916) on the "contattilita vivacissima" of the excretory channels in the posterior salivary glands of Octopoda (*O. macropus*, *O. vulgaris*, and *Eledone moschata*), for there exists no discontinuity between the glandular tubules and the excretory channels.

Perhaps it is not without interest to note, moreover, in the case of the posterior glands, that detection of carboxylic esterase activities and, more particularly, of acetylnaphtholesterase activities, is hindered by a superabundant discharge of gaseous bubbles that accumulate in the histological preparation so massively that they prevent examination and make fixation, by 10 per cent formaldehyde, for example, necessary. Since fixation suppresses this gaseous discharge, the bubbling may be due to an enzymatic activity that could not manifest itself before the blockage of the acetylnaphtholesterase activity.

The Digestive Tract

Most authors, if not all, have admitted that the keratinized segment of the digestive tract is only a transit route for the food; as Sellier (1910) said, "on ne rencontre aucune glande dans ses parois, en sorte que la digestion s'y effectue grâce aux liquides apportés des glandes annexes."*

It seems probable that the histochemically detectable phosphatase activities (alkaline and acid phosphomonoesterase, 5-nucleotidase, adenosine-triphosphatase) and the acetylnaphtholesterase activities (FIGURES 5*a* and *b* and 6*a*), are essentially implicated in the elaboration of the cuticle. However, it is possible that exchange of water, ions, and small molecules through this cuticular coating may occur, for the keratinized segments of the stomachs of ruminants (the first three pockets) are much more keratinized and, obviously, the site of such exchanges. Moreover Cohnheim (1902), who worked *in vitro* with *Octopus* and *Eledone*, placed digestive tracts in oxygenated blood of these animals and introduced into the lumens a known mixture of peptones and studied the composition of the blood at different intervals. He saw the blood become increasingly enriched by amino acids during the duration of the experiment and concluded that the wall of the digestive tract of these Octopoda has the power to liberate the amino acids from peptones.

We know, thanks to the observations of Romijn (1935), that the Decapoda, and more precisely *Sepia officinalis*, have an intestinal secretion able to hydrolyze casein, chloracetyltyrosine, leucyldiglycine, and glycylglycine; the secretion contains a proteinase and, more particularly, one carboxypeptidase, one aminopeptidase, and one dipeptidase. No equivalent data are at our disposal for Octopoda; it shall be, evidently, of paramount interest to locate precisely, with the techniques of histochemical detection, these peptidase activities and to elucidate the factors that control their variations. Bidder (1950 and 1957) drew attention to the specific enzymatic differences and said that "until more information is available it is clearly dangerous to combine, as has too often been done, one observation from *Octopus* with another from *Sepia* under the generalization 'in cephalopoda' "; Erspamer and Boretti (1950) are of the same opinion.

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* "One does not encounter any glands in these linings of the type used by the digestive system for easy transport of liquids from the adjoining glands." (Editor's translation.)

The butyryl- and acetylthiocholinesterase activities of the muscular tunics of the intestine (FIGURES 7*a*, *b*, and *c*) and of their ganglionic elements (FIGURES 7*b* and *c*) are probably implicated in regulation of the motive force of the digestive tract.

Kruger (1929) observed a carboxylic esterase activity in the digestive tract of *Octopus vulgaris*; it seems probable that the greatest part of that activity comes from the precaecal glandular complex. In effect, on the one hand, this complex is rich in acetylnaphtholesterase (FIGURE 8*b*) and in cholinesterase (FIGURE 8*c*) and, on the other hand, the nonkeratinized segment of the digestive tract is relatively poor in histochemically detectable carboxylinesterase activity (FIGURE 6*b*). This segment seems to be the elective site of absorption; it is also at this point that the infestation of *Octopus* by *Aggregata octopiana* Schneider (Protozoa: Coccidia) is the most important. Only in cases of massive infestation may spores of *Aggregata* be found all along the digestive tract, including the esophagus (FIGURES 4*a* and 6*a*); when the infestation is of the customary type, *Aggregata* is electively localized in the chorion of the nonkeratinized segment of the digestive tract. It is logical to assume that the choice of this habitat is conditioned by the abundance of nutritive material moving through the area.

The Precaecal Glandular Complex

The two glands that constitute this complex are equally rich in acid phosphomonoesterase and in acetylnaphtholesterase activities (FIGURES 8*a* and *b*), but they differ in all other enzymatic activities that have been studied here; thus, while the epithelium of the anterior caecal gland and the platelets elaborated by this epithelium strongly hydrolyze butyryl- and acetylthiocholine (FIGURES 8*c* and 10), the posterior caecal gland does not possess this power (FIGURE 10) except in some elements that constitute a network situated between the secretory tubules. The anterior caecal part lacks such fibrous elements capable of hydrolyzing acetylcholine, and it is exceptional to find similar elements in the anterior caecal gland, where they are exclusively satellites of the blood vessels, capable of hydrolyzing acetylthiocholine.

The enzymatic equipment of the two precaecal glandular formations thus appear very dissimilar; however they are equally rich in reducing groups of SH-types, in Sudanophilic lipids, and in polysaccharides.

The divergent conceptions among authors of the last century on the so-called "hepatic" or "pancreatic" role of these glands may be explained by the fact that all the products elaborated by the anterior caecal gland traverse the entire posterior caecal gland (FIGURES 1*a* and *b*); the secretions synthetized by the two glands are thus mixed together and discharged into the caecum by a single excretory channel.

Histochemical tests are not in accordance with the Castaldi and Musio hypothesis (1928), which ascribes to the posterior caecal gland some excretory function that is complementary to that of the kidney.

CONCLUSIONS

In the digestive tract of *Octopus vulgaris* Lamarck, the deshydrogenase activities that can be detected on fresh tissues are more important than the oxidase activities.

The anterior salivary glands are deficient in oxidases and in esterases, where it is a question of phosphatases or carboxylic esterases.

The posterior salivary glands possess varied enzymatic equipment, including succinodeshydrogenase, phosphomonoesterase, adenosine-triphosphatase, acetylnaphtholesterase, and cholinesterase activities.

The anterior keratinized segment of the digestive tract has phosphomonoesterase and adenosine-triphosphatase activities only in the subcuticle epithelium. These enzymes probably play some role in the elaboration of the cuticle, but their participation in ionic and molecular transfers through the epithelium cannot be excluded. The acetyl- and butyrylthiocholinesterase activities are electively concentrated in the muscular tunics and in the nerve ganglia; they seem implicated in the motive forces of the digestive tract.

The esterase activities of the nonkeratinized segment of the digestive tract predominate in the apical part of the epithelial cells; it is at this level that digestion and absorption probably prevail. The elective localization in that segment of *Aggregata Octopiana* Schneider, in the parasite-infested *Octopus*, seems to corroborate this observation.

The anterior and posterior caecal glands are rich in reducing agents, in Sudanophilic lipids, and in polysaccharides, as well as in acid phosphomonoesterase and in acetylnaphtholesterase. Moreover, the anterior caecal gland is rich in acetyl- and in butyrylthiocholinesterases, while the posterior caecal gland lacks these enzymatic activities at its epithelium. The base of the latter's tubes is surrounded by fibers that hydrolyze the acetylcholine, while the anterior caecal gland has only a few of these fibers, which are exclusively localized in the immediate area of the blood vessels.

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